UNIVERSIDADE DO MINHO
ESCOLA DE ENGENHARIA
GRUPO DE INVESTIGAÇÃO 3B's - Biomateriais, Biodegradáveis, Biomiméticos
DEPARTAMENTO DE ENGENHARIA DE POLÍMEROS

A Bone tissue engineering strategy based on starch scaffolds and bone marrow cells cultured in a flow perfusion bioreactor

Maria Manuela Estima Gomes

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To Luís

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Curriculum Vitae

Manuela E. Gomes was born in Águeda (Portugal) in 1973. She was graduated in Metallurgical and Materials Engineering at the Faculty of Engineering of the University of Porto in July 1997. In the last year of graduation she started her research work on Biomaterials which she continued after graduation, in a training period at Isotis, Bilthoven, Holland, (Locally supervised by Prof. Clemens van Blitterswijk and Dr. Joost de Bruijn). In 1999 she finished her thought part of the Master on Polymer Engineering at the School of Engineering of the University of Minho. In the same year she signed a contract with the University of Minho to work, under the responsibility of Dr. Rui L. Reis, in the European Project *ISOBONE* (Brite/Euram III): *A tissue engineering living bone equivalent, at the* Department of Polymer Engineering.

In 2001 she obtained her Master-degree in Polymer Engineering at the University of Minho. Her MSc. thesis was focused on the development and characterization of tissue engineering scaffolds based on starch polymers. She has been a researcher of the 3B's Research Group - Biomaterials, Biodegradables, Biomimetics, since this Group was created in the University of Minho, being co-responsible for the implementation of the internal Quality Assurance System. Furthermore, she has been supporting the lecturing of practical classes on Biomaterials to Applied Biology students (School of Sciences, Univ. of Minho) since 1999 and she has co-supervised several undergraduate students during their final graduation projects. She was also involved in the organization of two NATO/ASI courses, one on Tissue engineering (2001) and another on Biomineralization (2003) carried out in Alvor, Portugal. Manuela E. Gomes was also involved in the preparation of the proposal of the European Network of excellence EXPERTISSUES, coordinated by the 3B's Research Group, as well as other European (FP6) and National (Portuguese Foundation for Science an Technology) research project proposals.

She started her PhD in 2001 at the 3B´s Research Group-Univ. Minho (under the supervision of Dr Rui L. Reis), in cooperation with the Department of Bioengineering at the University of Rice (Houston, TX, USA) under the local supervision of Professor Antonious G. Mikos.

As a result from her research, she has participated in the most relevant international conferences of the Biomaterials field, with a total of 38 communications (poster and oral presentations). As a result of the work reported in this thesis, she was awarded twice with the *Student Travel and Professional Development Honorable Mention by the Society for Biomaterials (the first time in 2001*, Saint Paul-Minnesota, EUA and the second in 2003, Reno, Nevada, EUA). In 2003 she was also nominated for the Outstanding PhD student Award. In 2004 she received the *ResMed International Travel Scholarship* in the *7th World Biomaterials Conference* (Sydney, Australia) for the best work presented as oral communication. Manuela E. Gomes is author of 12 papers in international refereed journals (10 published and two submitted), including 2 review papers and 1 assay, and 13 book chapters.

This thesis is based on the following publications:

Papers in international refereed journals:

ME Gomes, RL Reis, AM Cunha, "*Alternative Tissue Engineering Scaffolds based on Starch: Processing Methodologies, Morphology, Degradation Behaviour and Mechanical Properties",* Materials Science and Engineering: C Biomimetic and Supramolecular Systems, 2002, 20:19-26

ME Gomes, RL Reis, "*Biodegradable Polymers and Composites in Biomedical Applications. From Catgut to Tissue Engineering. Part II: Systems for Temporary Replacement and Advanced Tissue Regeneration"*, International Materials Reviews, 2004, 49: 274-285

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ME Gomes, AJ Salgado, RL Reis, *"Bone Tissue Engineering Using Starch Based Scaffolds Obtained by Different Methodologies"*, in Polymer Based systems on Tissue Engineering, Replacement and Regeneration, RL Reis and D Cohn (Eds), NATO/ASI Series, Kluwer Press, Drodercht, 2002, 221-249.

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Resumo

Actualmente o transplante de tecido do próprio doente ou de um dador continua a ser a técnica mais utilizada para tratar defeitos ósseos provocados por doenças ou acidentes. No entanto, esta prática apresenta sérias limitações devido à escassez de dadores, ao risco de transmissão de doenças e/ou de rejeição imunológica e também devido ao problema da lesão dos tecidos envolventes que normalmente ocorre no local de onde é removido o tecido para transplante. O elevado número de pessoas em todo o Mundo que são afectadas por estes problemas, bem como os consequentes custos sócio-económicos, são razões acrescidas para a necessidade de desenvolver terapias alternativas para tratar a perda ou mau funcionamento de tecido ósseo.

A Engenharia de Tecidos é uma área científica em contínua expansão. Os desenvolvimentos conseguidos por esta área têm contribuído significativamente para diversos avanços no campo da Medicina Regenerativa. Esta ciência interdisciplinar combina os conhecimentos de diversas outras áreas, tão distintas como a Engenharia de Materiais e a Biologia, com o objectivo de desenvolver substitutos sintéticos para tecidos humanos. Para se atingir este objectivo utilizam-se, de uma forma genérica, combinações específicas de células e de um material de suporte tridimensional com propriedades adequadas, gerando um material híbrido cujas características podem ainda ser moduladas através do sistema de cultura usado.

A presente tese é centrada no desenvolvimento de estratégias de engenharia de tecido ósseo baseadas na cultura *in vitro* de células previamente "semeadas" num suporte tridimensional *("scaffold"*). Esta estratégia permite que as células adiram ao suporte, proliferem e segreguem matriz extracelular específica do tecido ósseo, até se obter um substituto artificial funcional com características do tecido original, que pode finalmente ser transplantado para tratar o defeito em causa. Para que uma estratégia deste tipo seja bem sucedida, pelo menos três componentes fundamentais devem ser cuidadosamente estudados: o material de suporte (*scaffold)*, as células a usar e o sistema de cultura *in vitro*. Daí que os principais objectivos desta tese estejam relacionados com estes três aspectos, nomeadamente:

- Desenvolvimento de *scaffolds* biodegradáveis a partir de polímeros à base de amido de milho que induzam a adesão e proliferação celular e que apresentem propriedades adequadas, tais como a porosidade e interconectividade entre poros, de forma a proporcionar um ambiente que favoreça o desenvolvimento *in vitro* de um material híbrido com características similares ao osso humano.
- Estudo da utilização de células da medula óssea como uma potencial fonte de células para engenharia do tecido ósseo, uma vez que estas células podem ser facilmente recolhidas do próprio paciente a tratar por métodos não-invasivos (biopsia) e em

quantidades suficientes. Além disso, tratando-se de uma fonte de células autólogas (obtidas do próprio paciente) permitem evitar os riscos de transmissão de doenças contagiosas e/ou de rejeição pelo sistema imunológico.

- Estudo da influência das condições de cultura *in vitro* geradas por um bioreactor de perfusão (em comparação com os métodos tradicionais de cultura em condições estáticas) no desenvolvimento dos materiais híbridos, compostos pelas células e *scaffolds*, assim como as interacções do ambiente proporcionado por este sistema de cultura com as diferentes estruturas/arquitecturas e porosidades dos *scaffolds* utilizados.

Estes objectivos convergem para o objectivo geral desta tese que consistiu no desenvolvimento de uma terapia de engenharia do tecido ósseo alternativa ás existentes e com potencial para vir a ser posteriormente utilizada na prática clínica. Este objectivo foi avaliado através do estudo da funcionalidade dos materiais híbridos obtidos em diferentes condições de cultura *in vitro* (e utilizando diferentes *scaffolds*), partindo do principio que o sistema de perfusão poderia eventualmente superar as limitações de difusão típicas dos sistema de cultura estática e simultaneamente proporcionar estímulos mecânicos ás células, semelhantes aos encontrados em condições fisiológicas.

O trabalhou desenvolvido permitiu propor várias metodologias de processamento que conduziram à obtenção de *scaffolds* com propriedades e estruturas porosas muito interessantes. De um modo geral, estes *scaffolds* permitem a adesão, proliferação e diferenciação das células de medula óssea, quando cultivadas em condições estáticas ou no bioreactor de perfusão. Foi demonstrado que a estrutura porosa dos *scaffolds* e especialmente a interconectividade entre poros, afecta a homogeneidade do tecido formado. A porosidade dos *scaffolds* influencia o desenvolvimento sequencial das células osteoblásticas e, em combinação com as condições de cultura usadas pode influenciar a funcionalidade dos "tecidos" formados *in vitro.*

Os resultados obtidos salientam a importância do sistema de cultura utilizado em estratégias de engenharia de tecido ósseo como a que é proposta nesta tese. De facto, o bioreactor de perfusão contribui significativamente para melhorar a funcionalidade dos materiais híbridos/tecidos desenvolvidos *in vitro*, uma vez que combina factores biológicos e mecânicos que proporcionam um melhor desenvolvimento das células contidas no *scaffolds,* conduzindo á obtenção de um tecido mineralizado semelhante ao osso humano. Os resultados obtidos demonstram que a cultura de células de medula óssea em *scaffolds* biodegradáveis á base de amido de milho num bioreactor de perfusão, pode também constituir um modelo para o estudo de mecanismos biológicos associados ao processo de formação de tecido ósseo, o que por sua vez pode contribuir para a avaliação e melhoria de estrategias de engenharia de tecidos do osso.

Abstract

There is a very significant and well-known clinical need for the establishment of alternative therapies for the treatment of bone tissue loss or failure resulting from injury or disease, as the transplantation of tissues in these patients is severely limited by donor scarcity and is highly associated to the risk of immune rejection and disease transfer. The always evolving field of tissue engineering has brought a number of significant advances to regenerative medicine. This interdisciplinary science combines the knowledge and experience of many different fields, from materials science to biology, in order to develop tissue-like substitutes. This is generally achieved through a specific interplay between cells and scaffolds (and in some cases, growth factors), which can also be modulated by the culturing system used.

This thesis focuses on bone tissue engineering approaches based on culturing cells-scaffold constructs *in vitro*, allowing the seeded cells to proliferate and secrete tissue specific extracellular matrix (ECM) until obtaining a functional tissue-like substitute that can be transplanted to the tissue defect to be treated. To achieve the success of such tissue engineering approach, there are at least three key issues that must be carefully studied: the scaffold material, the cells and the culturing environment. Therefore, the main objectives proposed for this thesis address these three aspects:

- Development of appropriate starch-based scaffolds capable of inducing the attachment and proliferation of the seeded cells, and exhibiting adequate properties, such as porosity and pore interconnectivity, in order to provide an appropriate environment for the *in vitro* development of bone-like tissue.
- Studying the use of bone marrow cells as a reliable cell source for bone tissue engineering application, as that can be readily available (in sufficient amounts) and obtained by simple procedures (biopsy) from the same patient, avoiding the risk risks of disease transmission and/or immune rejection.
- Studying the influence of *in vitro* culturing conditions, namely flow perfusion, on the development of cell-scaffolds constructs, as well as the interactions of the environment provided by this culturing method with different scaffolds architectures and porosities.

These objectives converge to the main goal of this thesis, which is the development of an improved bone tissue engineering therapy. This was assessed by the functionality of the tissue engineering constructs obtained under different *in vitro* culturing conditions (and from different scaffolds), in the light of using flow perfusion bioreactor having the potential to mitigate diffusion limitations typical of static culturing and simultaneously provide physiological-like stimulus to the seeded cells.

This work allowed for the development of a range of processing methodologies leading to scaffolds with different properties and porous structures, also depending on the synthetic component of the starch-based polymeric blend.

In general, these starch-based scaffolds allowed for the adhesion, proliferation and differentiation of marrow stromal cells towards the osteoblastic phenotype, under static and flow perfusion conditions. It was demonstrated that scaffold architecture and especially pore interconnectivity affect the homogeneity of the formed tissue. The scaffolds porosity influences the sequential development of osteoblastic cells and in combination with the culture conditions may affect the functionality of *in vitro* formed tissues.

The work developed also emphasized the importance of the culturing system in bone tissue engineering approaches such as the one proposed in this thesis. Flow perfusion augments the functionality of scaffold/cell constructs grown *in vitro* as it combines both biological and mechanical factors that enhance cell differentiation and cell organization within the construct, towards the development of bone-like mineralized tissue. Additionally, this study also shows that flow perfusion bioreactor culture of marrow stromal cells combined with the use of appropriate starch based biodegradable scaffolds may also constitute a useful model to study bone formation and assess bone tissue engineering strategies *in vitro*.

List of abbreviations

A

AA: Acrylic acid
ALP: Alkaline phosphataze activity

B

BA1: Blowing agent 1
BA2: Blowing agent 2
BA3: Blowing agent 3
BPO: Benzoic peroxide
BMP: Bone morphogenetic protein

C

Ca: calcium
CAD:computer aided design

D

DMOH: N – dimethylaminobenzyl alcohol

E

ECM: Extracellular matrix
EtO: Ethylene oxide

F

FGF-2: Fibroblast growth factor
FTIR-ATR: Fourier Transformed Infrared Spectroscopy with Attenuated Total Reflectance
FDM: Fused Deposition Modeling
FCS: Fetal Calf Serum

H

HA: Hydroxyapatite
HARV: High Aspect Ratio
H&E: Hematoxilin and eosin

I

IGF: Insulin Growth Factor

M

MEM: Minimal Essential Medium
µCT: Microcomputerized tomography

O

OC: Osteocalcin
OP: Osteopontin

P

PHB: Polyhydroxybutyrate
PGA: Poly (glycolic acid)
PLA: Poly (lactic acid)
PLG: Poly(D,L-lactide-*co*-glycolide)
PLLA: Poly (L-lactic acid)
PCL: Poly (ε - caprolactone)
PDS: Poly (dioxanone)
PEO: Polyethylene oxide

PBT: Polybutylene teraphthalate
PPF: Poly (propylene fumarate)
PVA: Poly (vinyl alcohol)
PDGF: Platelet-derived Growth Factor
PBS: Phosphate buffer solution
PET: Polyethylene teraphthalate
PTFE: Polytetrafluoroethylene

R

RP: Rapid prototyping
RWV: Rotating wall vessel
RBMC: Rat bone marrow cells

S

SCA: Blend of corn starch with cellulose acetate
SEVA-C: Blend of corn starch with ethylene vinyl alcohol
SPCL: Blend of corn starch with polycaprolactone
SPLA: Blend of corn starch with polylactic acid
SEM: Scanning electron microscopy
SLTV: Slow lateral turning vessel

T

TGF: Transforming Growth Factor
TF-XRD: Thin-film X-ray diffraction
Tg: Glass transition temperature
Tm: Melting temperature

V

VEGF: Vascular Endothelial Growth Factor

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Chapter I

GENERAL INTRODUCTION

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Tissue engineering offers the possibility to help in the regeneration of tissues damaged by disease or trauma and, in some cases, to create new tissues and replace failing or malfunctioning organs.[1-7] This is achieved through the use of degradable biomaterials to either induce surrounding tissue and cell ingrowth or to serve as temporary scaffold for transplanted cells to attach, grow, and maintain differentiated functions.[3-5,7-17] In any case, the role of the biomaterial scaffold is temporary, but crucial to the success of the strategy. Cells and culturing methodologies are also critical issues in bone tissue engineering approaches. Finally it is important to recognize the characteristics and mechanisms of this tissue as this information is crucial to help designing improved combinations of scaffolds, cells and culturing methodologies towards the ultimate goal of bone tissue engineering, i.e., the development of bone-like substitutes.

*** This chapter is based on the following publications:**

ME Gomes, RL Reis. *Biodegradable Polymers and Composites in Biomedical Applications. From Catgut to Tissue Engineering: Part II- Biodegradable polymers in temporary replacement and advanced tissue regeneration.* International Materials Reviews (2004) 49: 274-285

ME Gomes, AJ Salgado, RL Reis. *Bone Tissue Engineering Using Starch Based Scaffolds Obtained by Different Methodologies.* In Polymer Based Systems on Tissue Engineering, Replacement and Regeneration, Eds: RL Reis and D Cohn, NATO/ASI Series, Drodercht , Kluwer Press (2002) 221-249.

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1. TISSUE ENGINEERING APPROACHES FOR THE REGENERATION OF BONE

1.1. Bone tissue

Bone is a dynamic tissue which undergoes remodeling as it is constantly being resorbed and rebuilt (or formed), following injury.[18-20] In spite of being capable of self-regeneration after injuries or to remodel in relation to local stresses, in case of severe injury, the fracture repair mechanism of bone can fail and thus bone does not heal correctly and does not regain its mechanical function [3,7,20-24]. Such clinical scenarios are known as fibrous non-unions and require additional treatment to aid the healing process.[7]

Moreover, bone tissue can suffer from several diseases, most of which are due to an imbalance between the breakdown and formation of bone resulting in systemic or local bone loss. These include: osteoporosis, hyperparathyroidism, hypercalcemia and malignancy, Paget's disease, metastatic disease in bone, local destruction of bone in the jaws in periodontal disease, periarticular bone loss in rheumatoid arthritis, bone loss in immobilization due to the absence of mechanical stimuli, and bone loss associated to glucocorticoid treatment.[20] All of these conditions could be helped by blocking bone resorption, but this is not always possible. In such situations, bone tissue engineering can bring up the ultimate solution and hope for many patients suffering from these problems.

For developing tissue engineering strategies to regenerate bone, it is productive to frequently review bone anatomy[22,25-27] and to revisit the concepts of bone formation and repair. Understanding the mechanisms of bone formation as well as the manner by which this extraordinary tissue repairs or regenerates, considering the contribution of each its natural components and its functions, should be the basis for attempting to develop new strategies for bone tissue regeneration [27,28].

1.1.1. Overview of bone biology

Bone is a natural composite material containing approximately 60% mineral, 10% water and 30% collagenous matrix.[29] This fascinating material provides at least four principal functions in the human body. The first is that bone provides a structural framework, against which the muscles can contract to move the body and against which the various organs can maintain their relative position in the body.[7,22,23,30] Another function of the skeletal system is to provide a mineral (predominantly calcium and phosphate) reservoir for the rest of the body.[22,23,30] A third principal function of the bone is to provide protection for the

most vital internal organs.[22,23,30] A fourth function of the bone is to provide milieu (via marrow) [22,23] for the development of the immune system. To fulfill these functions, bone is continuously broken down and rebuilt.[20,31] Resorption is carried out by osteoclasts that dissolve bone mineral and digest bone matrix and which are derived from the bone marrow mononuclear cells (preosteoclasts) that line the bone-forming surfaces.[20,21,30,32] Bone resorption takes place at a specialized area of the osteoclastic cell membrane called the "ruffled border".

Osteoblasts are the bone-forming cells.[20,21,30,33] They are cuboidal, plump cells that are organized in layers and act in coordinated fashion to generate the matrix.[20] They work together to produce the lamellar structure characteristic of bone. Osteoblasts secrete the bone matrix, which subsequently proceeds to mineralize extracellularly.[20,30,34] Osteoblasts originate from mesenchymal stem cells/precursor cells in response to an inducing event and undergo differentiation, a stepwise process that can be defined by a temporal sequence of expression of genes encoding osteoblast phenotype markers divided in three distinct periods: a growth period (proliferation), a period of matrix development and a mineralization period.[35] During this process, the cells start producing characteristic products of osteoblasts, including all the constituents of extracellular matrix, such as type I collagen, which constitutes 90% of the matrix and noncollagenous proteins, such as osteopontin, osteonectin, bone sialoprotein, biglycan, matrix gla protein, decorin, and osteocalcin.[20,36] Osteoblasts are also very rich in alkaline phosphatase, which participates in the mineralization process. Osteoblasts deposit about 0.5μm of matrix per day and their forming periods last about 100 days. Some of the osteoblasts are then buried within the matrix which they formed and are called osteocytes, other become flattened cells on the surface of the bone and are called lining cells.[20,30] Osteocytes communicate with each other through projections, which join in gap junctions and may provide communication across the bone. [20] This brings up the second function of osteoblast lineage cells, which is the regulation of bone metabolism and remodeling. Osteoblastic cells respond to several bone resorptive stimuli (including, for example, parathyroid hormone) and also to a significant number of growth factors (and produce some of them).[20]

Macroscopically, the bone of the mature skeleton consists of cortical (or compact) bone (80%) and cancellous (or trabecular) bone (20%).[19,22,30] Compact bone is distinguished from trabecular bone by the spatial orientation of its mineral and organic components, and by its characteristic locations in the skeleton. Compact bone comprises the outer tubular shell of the long bones, and the outer surface of the small bones and flat bones. It is much more dense than trabecular bone, and it consists of parallel cylindrical units called osteons (or Harversian systems). Compact bone is anisotropic, and the orientation of the osteons

determines, to a great extend, the directionality of its mechanical properties.[19,22] Trabecular bone is less dense than compact bone and is comprised of an array of plates and rodes of bone tissue and forms an open-cell foam. The orientation of the plates and rodes of the bone is such that the trabecular bone is also anisotropic. It optimizes resistance to usual stresses that occur in its particular location.[19,22]

1.1.2. Bone formation and calcification

Bone formation occurs by intramembranous or endochondral processes. In the intramembraneous process, bone formation begins when mesemchymal progenitors condense (form clusters of cells) and differentiate directly into osteoblasts, while in the endochondral ossification process the same progenitors first form a cartilage template that is later replaced by bone.[22,30,37] Intramembraneous ossification is mainly responsible for the development of flat bones from the skull and for the addition of bone on the periosteal surfaces of long bones [22,37]. Endochondral ossification occurs in the formation of long bones, vertebrae and fracture repair.[22,37] Besides the different processes on bone formation, also distinct embryonic lineages are involved in forming the different parts of the skeleton. A third type of bone development, called appositional formation, occurs during enlargement of bone and during remodeling.[38] In this case, osteoblasts attach to existing bone and secrete matrix, often in layers. All three types of formation occur constantly and a particular bone can be formed through any combination of these developmental schemes.[38]

Independently of the bone formation process that occurs, the collagen matrix secreted by the osteoblasts undergoes mineralization.[22,35,38-40] As osteoblasts are separated by calcifying matrix, they are entrapped in spaces called the lacunae. These entrapped osteoblasts - the osteocytes – gradually loose their ability to produce matrix.[20,22] These cells communicate with other osteocytes via long processes (canaliculi), which are organized before calcification. There is a delay between the formation of the matrix and its mineralization, which is believed to occur so that there is spatial separation between calcified bone and the overlaying cells. Matrix mineralization occurs in two steps correspondent to the nucleation and growth of calcium phosphate crystals. The nucleation can be homogenous or heterogeneous.[22,38] In homogenous nucleation, the formation of crystal is due to supersaturation of the local environment with the appropriate ions. The heterogeneous nucleation occurs only at surfaces where the interaction between the surface and the ions lowers the interfacial energy requirement so that nucleation can

proceed at concentrations that are less than that of supersaturation. After nucleation, amorphous calcium phosphate may be the first to precipitate, which is then converted into octacalcium phosphate and finally to hydroxyapatite.[38]
As woven bone is formed and calcified, it is remodeled to form mature lamellar bone[22]. On a larger scale, both woven and lamellar bone can be found in either trabecular or cortical bone. Lamellar bone is generated more slowly than woven bone and is less mineralized.[20,22]

1.1.3. Bone formation and regeneration: the importance of the mechanical environment

Bone has enormous capacity for growth, regeneration and remodeling. The mechanical environment is one of the major factors involved in these processes. Mechanical loading of physiological relevance magnitudes has been shown directly to initiate bone modeling in animal models,[41-45] although this is not the only factor contributing to these changes.[46] In contrast, lack of load has been shown to promote tissue atrophy and bone loss.[41,43] In fact, during growth and development, the skeleton optimizes its architecture by subtle adaptations to the thousands of repetitive mechanical loads to each it is exposed daily. The mechanisms for adaptation involve a multistep process of cellular mechanotransduction[44,46-48] that includes: i) mechanocoupling: conversion of mechanical forces into local mechanical signals, such as fluid shear stress, that initiate a response by bone cells; ii) biochemical coupling: transduction of a mechanical signal to a biochemical response involving pathways within the cell membrane and cytoskeleton; iii) cell-to-cell signaling from the sensor cells (probably osteocytes and bone lining cells) to effector cells (osteoblasts and osteoclasts) using prostaglandins and nitric oxide as signaling molecules[46,47,49]; and iv) effector response: either bone formation or resorption to cause appropriate architectural changes. These architectural changes tend to adjust and improve the bone structure to its prevailing mechanical environment[47].

The repair and regeneration of bone can be characterized as a symphony of cellular activity [50,51] beginning with an acute inflammatory response followed by granulation tissue infiltration, recruitment, proliferation and differentiation of osteogenic cells, matrix formation and mineralization, and eventual remodeling. The process occurs within the context of biological and mechanical signals. Biologically, the regulation of the repair is accomplished through the expression of a cascade of growth factors, hormones, and cytokines[50,51] that condition the wound and directly influence cell migration, proliferation, differentiation and synthesis. Similarly, mechanical forces transmitted via the extracellular matrices being

assembled, influence the patterning of this cellularly orchestrated activity. From a hierarchical perspective, the success of the repair or regeneration of bone will depend on the regeneration of the necessary molecular signaling cascades, the availability and viability of progenitor cells, nutritional support, and appropriate mechanical environment.[50,51]

In summary, the goal of a bone tissue engineer is to exploit the body's natural ability to repair injured bone with new bone tissue.[22,27,52] In general, bone tissue engineering strategies should attempt to provide the reconstruction region with appropriate initial mechanical properties, encourage new bone to form in the region, and then gradually degrade to allow the new bone to remodel and assume the mechanical support function.[22,27]

1.2. Tissue engineering strategies

Conventional approaches in bone repair have involved biological grafts such as autogenous bone or autografts,[53-55] allogenic bone or allografts[53,56] and xenografts.[53] Currently, autografts are the most often used in clinical settings, as they do not represent risk of immune rejection or disease transfer.[53,54,57] However, the limited availability of autografts and the risks of morbidity have fueled the continuously growing interest in the development of alternative approaches to bone repair based on tissue engineering strategies.[27,53,55,57,58]

As stated before, the ultimate goal of tissue engineering is to replace, repair or enhance the biological function of damaged, absent or dysfunctional elements of a tissue or an organ. This goal is accomplished using cells that are manipulated through their extracellular environment to develop engineered tissues that can function as living biological substitutes for tissues that are lacking.[59] Many different strategies may be used to develop these engineered tissues. The selection of the best strategy for developing hybrid materials for the regeneration of a specific tissue defect is determined by several factors, such as the technical feasibility, required properties of the implant and the interaction of the host with the graft.[59] Basically, three general strategies (which are schematically represented in Figure I.1) have been adopted for the creation of new tissue[60-64]. These strategies are described bellow.

1.2.1. Cell self-assembly

The *Cell self-assembly* approach corresponds to the direct *in vivo* implantation of isolated cells or cell substitutes[60-65] and its based on cells synthesizing their own matrix. This approach avoids the complications of surgery, allows replacement of only those cells that supply the needed function and permits manipulation of cells before infusion. Its potential limitations include failure of the infused cells to maintain their function in the recipient, and immunological rejection.[60-62] For other authors[66] this approach involves a layer of cells secreting their own matrix, which over a period of *in vitro* culturing becomes a sheet and the formation of multiple layers like can eventually result in the formation of skin substitutes or blood vessels, for example. However, it is well known that many cell types are *anchorage dependent* (their function is dependent upon specific cell-substrate interactions), and therefore their direct transplantation or *in vitro* culturing without a scaffold, as suggested in this approach, results is cell death or loss of function.[24,61]

1.2.2. Acellular scaffold

This approach is based on the direct *in vivo* implantation of biomaterials[24,61-65,67,68] and relies on the ingrowth of tissue and cells into a porous material; this process, by which the regeneration is affected by ingrowth from surrounding tissue, is known as *tissue induction*[24] In many cases, in this approach, the matrix (polymeric scaffold) is loaded with growth factors or any other therapeutic agent. With this approach, the issue of cell sourcing is eliminated but its success depends on the infiltration and recruitment of the appropriate type of cells from the body in order to populate the construct and thus facilitate a proper tissue repair. In the case of bone, this three-dimensional process depends on the chemical surface properties of the implant, its three-dimensional structure and porosity, and its rate and mechanism of degradation.[34] These are the properties of the materials that may enhance the attachment, migration and distribution of cells responsible for the bone-healing response throughout the volume of the graft site, i.e., the osteoconduction behaviour. When porous osteoconductive structures are implanted into or adjacent to bone, cells from surrounding tissues migrate into available void volume of the matrix. The process is characterized by an initial ingrowth of fibrovascular tissue and new blood vessels.[34,68] This tissue invades the void volume of the scaffold and is latter followed by bone formation.

1.2.3. Cell-seeded polymeric scaffolds

In this approach, the temporary scaffold provides an adhesive substrate for the implanted cells and a physical support to organize the formation of the new tissue.[24,60-65,67,69,70] Transplanted cells adhere to the scaffold, proliferate, secrete their own extracellular matrices (ECM), and stimulate new tissue formation. During this process, the scaffold gradually degrades and is eventually eliminated.[62,64,68-73]

This is considered by many as the classic tissue engineering approach and is the most widely studied for bone regeneration. This results mainly from the statement that all successful bone healing requires the presence of a sufficient amount of osteoblastic progenitor cells,[22,34,64,74] which is limited in many clinical settings. These settings include sites of large bone defects, sites containing extensive scar tissue from previous surgery or trauma, sites of previous infection or radiation, sites in which the bone may be diseased or sites with compromised vascularity.[34] There are also systemic conditions such as diabetes or metabolic bone disease and pharmaceutical agents such as nicotine, systemic glucocorticoids or chemeotherapy, that limit the number or function of progenitor cells.[20,34,74] In these cases, that are common in clinical practice, the implantation of an osteoconductive material alone or even an osteoconductive scaffold loaded with one or more osteoinductive growth factor may not be enough to induce a reliable and optimal bone-healing response.[22,34,64,74]

Therefore, the key for successful repair/regeneration of bone is to provide the repair site with sufficient osteogenic progenitor cells in a suitable scaffold to ensure osteoblastic differentiation and optimal biosynthetic activity of bone matrix and growth and differentiation factors. Exclusion of interfering tissues, promotion of vascular penetrance, and provision of appropriate mechanical and other instructional cues must be engineered. As one gains more information about the identity and reactivity of all the triggering factors, it may be possible to orchestrate massive bone regeneration by clever combinations of scaffolds and such instructional agents.[75]

The experimental work described in this thesis is based on the last approach that corresponds to *in vitro* culturing of cell-scaffolds constructs prior to implantation. Therefore, the reminder of this chapter will focus only on the three main issues involved in this approach: i) the polymeric scaffolds, ii) the cells sources and iii) the culturing system.

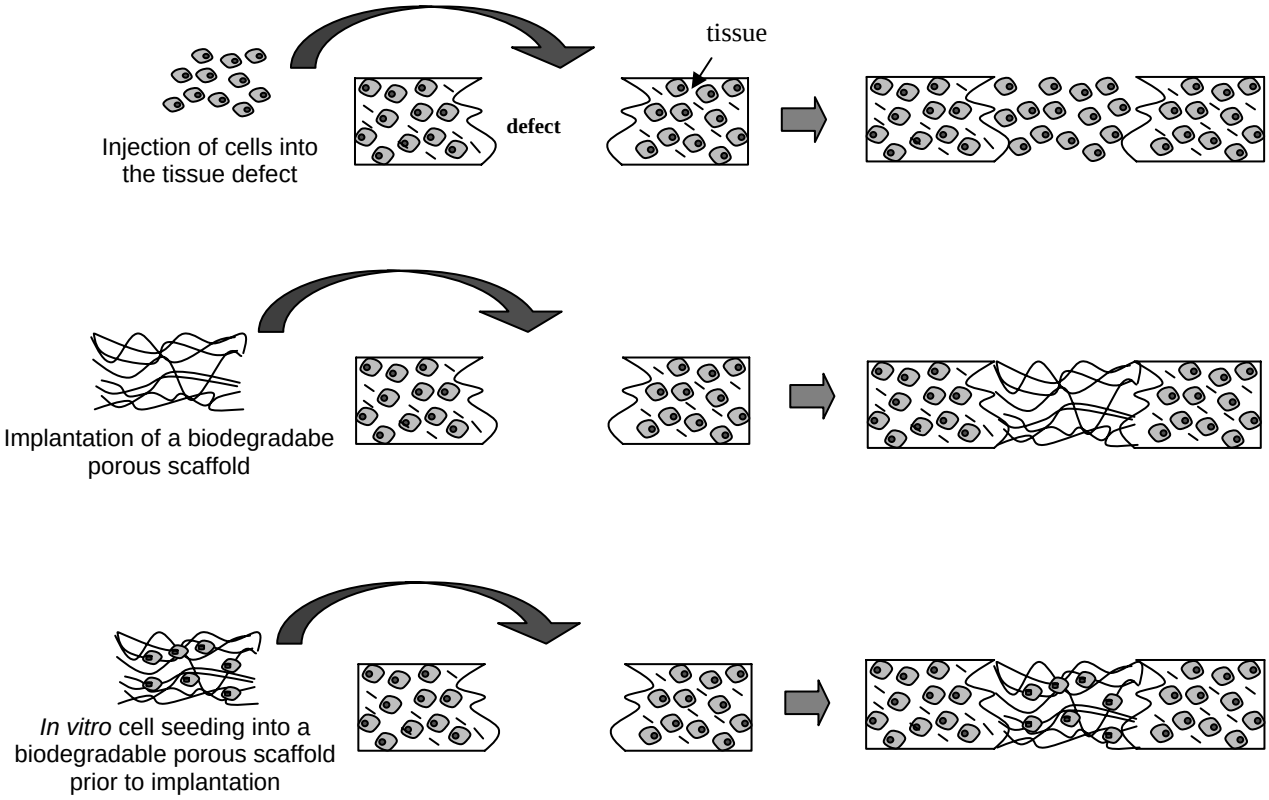

Figure I.1: Schematic representation of the three main generic tissue engineering approaches that may be used to regenerate a tissue defect. a) Cell self-assembly b) acellular scaffold c) cell-seeded polymeric scaffold.

2. DESIGN AND PROCESSING OF SCAFFOLDS FOR BONE TISSUE ENGINEERING

2.1. Scaffold requirements

The requirements for a scaffold material to be considered suitable for tissue engineering applications are complex and in many cases there is no consensus among the biomaterials research community about the specific demands that are required for a particular application. These requirements depend mainly on the tissue to be restored and on the location and size of the defect to be treated. Nevertheless, there are some general key characteristics that a scaffold material must possess:

i) biocompatibility[1,3,7,11,24,67,76-80] both in as-implanted and degraded form, i.e., the scaffolds and their degradation products should not invoke an adverse immune response or toxicity;
ii) appropriate mechanical properties[1,3,7,23,65,67,76-79,81-84] to provide the correct stress environment for the neo-tissue; this is particularly important for the regeneration of hard tissues, such as bone.
iii) *controlled degradation rate*[3,7,11,24,61,67,76,78,80,81,84]: because tissues regenerate at different rates, the degradation rates should be adjustable to match the rate of tissue regeneration, since strength decreases as the material degrades over time;
iv) *appropriate pore size and morphology*[1,3,7,11,24,61,67,78,79,81,84]: porosity, pore size and pore structure are important factors that are associated with nutrient supply to transplanted and regenerated cells. Small diameter pores are preferable to yield high surface area per volume, as long as the pore size is greater than the diameter of a cell in suspension (typically 10 μm). There is a lack of consensus regarding the optimal pore size for maximum tissue ingrowth and/or for an optimal cell growth, but is generally accepted that this depends on the tissue that is intended to restore/substitute. In the case of bone regeneration, some authors defend that a maximal tissue ingrowth is attained with a pore size ranging from 200 to 400 μm,[67] for others it should be from 100 to 150 μm,[3] or from 100 to 350 μm,[85] for example. Interconnectivity between pores is highly desirable when compared to the isolated pores, since an interconnected pore network structure enhances the diffusion rates to and from the centre of the scaffold and facilitates vascularization,[1,3,7,24,67,78,79,81,84,86,87] thus improving oxygen and nutrient supply and waste removal.
v) *appropriate surface chemistry for cell attachment, proliferation and differentiation*[1,3,7,24,67,77-79,81,84,86,87]: because most organ-cell types are anchorage dependent, they require the presence of a suitable substrate to retain their ability to proliferate and perform differentiated functions since cell adhesion is the pre-requisite for further cellular functions, such as spreading, proliferation, migration and biosynthetic activity. Therefore, the surface characteristics of materials, whether their topography, chemistry, surface energy or wettabbility, play an essential role in cell adhesion on biomaterials.[88-90] However, it is very rare that any biomaterial with good bulk properties for a specific use in the biomedical field also possesses the required surface characteristics[91,92] for that application. It follows that most of the biomaterials need surface modification to acquire surface characteristics that allow for an adequate cell adhesion.[91,92] These surface modifications include, for example, roughening, coating, blending and grafting.[91,92]
vi) *easily sterilized* [24,76] either by exposure to high temperatures, ethylene oxide vapour, or gamma radiation and remain unaffected by one of these techniques.

vii) *easily processed into three-dimensional shapes of irregular geometry*[3,7,11,61,67,76-78] that can be maintained after implantation. In some cases, a scaffold with unique three-dimensional geometry is required to fit an irregular defect. The regenerated tissue is therefore expected to take the shape of the initial scaffold.

2.2. Scaffolding materials – the starch based polymers

In scaffold-based tissue engineering strategies, the successful regeneration of tissues from matrix-producing connective tissue cells or anchorage dependent cells (e.g. osteoblasts) relies on the use of a suitable scaffold. Therefore, the design and production of an appropriate scaffold material is the first, and one of the most important stages in hard tissue engineering strategies based on seeding and culturing an appropriate type of cell onto the constructs prior to implantation. In this critical stage, the selection of the most adequate raw material is a primary consideration. There are many biocompatible materials available among metals, ceramics and polymers. However, the criteria of biodegradability excludes the use of all metals and most ceramics as scaffolds materials.[3,24] Although biodegradable/bioresorbable ceramic materials, such as tri-calcium phosphate and sea coral, have been used with some success[3,24] as scaffold materials (mainly in orthopaedic applications), they usually present at least one of the following limitations: first they are usually not only quite brittle but also difficult to process into porous materials with complex shapes, and second, it is currently not possible to generate matrices with clinically useful degradation rates from most of the available ceramics. Polymers, on the other hand, are ductile and easily formed into any shape. Traditionally, in spite of the wide range of biodegradable polymers available (currently available natural and synthetic polymers and their properties can be found elsewhere[93]), there is a strong tendency to choose those that have history of regulatory approval, i.e., materials which were primarily developed for other applications, instead of letting the application guide the choice of the material.[77] For these reasons, the most widely used polymers in tissue engineering have been the poly(α-hydroxy acids) of the aliphatic polyesters, i.e. polyglycolic acid (PGA), polylactic acid (PLA) and copolymers (PLGA) of these materials. This has created additional difficulties for the development of new materials with improved properties, specifically tailored for tissue engineering applications.

It is believed, that the best biodegradable polymer for biomedical applications, including those related with tissue engineering, may be found taking steps towards the development of new biomaterials that combine the most favorable properties of synthetic and natural

polymers.[61,87,94] Biodegradable blends of corn starch with several synthetic polymers, including the ones produced by Novamont (Novara, Italy), have been originally proposed by the University of Minho researchers as potential alternatives to the commonly used biodegradable polymers, for a variety of biomedical applications.[95-144] These materials are based on blends of corn starch (in amounts varying from 30 up to 50 %wt) with poly(ethylene vinyl alcohol) (SEVA-C), cellulose acetate (SCA), poly(ε-caprolactone) (SPCL) and poly-lactic acid (SPLA).[95,145,146]

These polymers can be designed into distinct material formats and/or different properties by tailoring the synthetic component of the starch-based blend, their processing methods, and the incorporation of additives and reinforcement materials. These polymeric blends are degraded by hydrolytic processes and several enzymes[136,147-149] can also be involved in the process, mainly alpha-amylase, beta-amylase, alpha-glucosidase and other debranching enzymes.[136,148,149] The biocompatibility and non-immunogenecity of starch-based polymers has been well demonstrated by several *in vitro* [118,125,134,143,150-152] and *in vivo* studies[106,153]. For all these reasons, together with their low cost and abundance of raw materials, starch-based polymers have been suggested for a wide range of biomedical applications, such as, for example, partially degradable bone cements,[99,109,140-142] as systems for controlled release of drugs[98,103,104,109] as bone substitutes in the orthopaedic field [96,97,116,122,130] or as scaffolds for tissue engineering,[100,110,120,121,123,128,143,144,154] as it is also proposed in this thesis.

2.3. Scafolds processing methodologies

In order to meet all the necessary requirements, scaffold materials must be fabricated from polymers with adequate properties but the establishment of basic requisites and design constrains its not an easy task and requires a deep knowledge about all the materials features that can interfere with cells/tissues-scaffold interactions. The selection of the appropriate tissue engineering approach will help define the most adequate scaffold design and the correspondent required properties, which must be able to induce the desired tissue response.[59,155] Three-dimensional porous structures have been recognized as the most appropriate design to sustain cell adhesion and proliferation, although several specific applications in tissue engineering may take advantage of other design formats or combination of different materials designs.[77]

As discussed in previous section, besides the obvious demands of biocompatibility and biodegradability, an ideal tissue engineering scaffold should exhibit appropriate

mechanical properties[1,3,7,23,65,67,76-79,81-84] and a suitable degradation rate.[7,24,76,80,81,156] Furthermore, the scaffold must possess adequate porosity, interconnectivity and permeability to allow the ingress of cells and nutrients[7,80,81,156] as well as the appropriate surface chemistry for enhanced cell attachment and proliferation.[11,61,81,156] For most applications, tissue engineering scaffolds must provide cell anchorage sites, mechanical stability and structural guidance and, when implanted, provide an adequate interface to respond to physiological and biological changes in order to integrate with the surrounding native tissue. Taking into account these requisites, the processing technology used to produce the scaffolds is mainly expected to provide the maximal control over macro and microstructural properties of the scaffold without negatively affecting the other properties that provide good scaffold-cells interactions such as toxicity and/or surface chemistry/topography.[157] Other important requirements for a scaffold fabrication method include the process accuracy and reproducibility, i.e., the methods should be able to produce scaffolds with consistent properties and physical forms when using the same processing parameters.

However, the properties of the scaffold are also dependent on the natural or synthetic material selected for its production. For the selection of the most appropriate scaffold material for a given application, besides the intrinsic properties of the wide range of materials available, it is also important to consider that each material or combination of materials has a different degree of processability and processing requirements.

The development of matrices to serve as templates for cell attachment/suspension and delivery has progressed at a tremendous rate in the past years and a wide range of methodologies have been developed. Usually, these methods involve either melting or solvent casting processing. Melt processing involves heating the polymer above the glass transition temperature (Tg) or the melting temperature(Tm) and depends on melt viscosity. Solvent processing depends on polymer solubility in various organic solvents and on the solvent volatility.[3,24] Among these processing techniques are methods such as solvent casting and particulate leaching,[15,67,158-160] membrane lamination,[10,67] fiber bonding,[80,87,161-164] phase separation/inversion,[67,165] melt based technologies,[7,100,121,123,158] microparticles aggregation,[166,167] and micro-wave baking and expansion,[103] just to cite some examples. More recently highly reproducible 3-D scaffolds have been obtained using rapid prototyping technologies such as fused deposition modelling (FDM) and 3-D printing.[80,168-170]

The methodologies developed so far, have been applied to fabricate scaffolds for tissue engineering with different levels of success. In general, most of these methods present other main limitations such as[157]:

i) *Manual intervention*: most of the techniques available rely on manual-based procedures which are practically impossible to transform in industrial scaled-up methods. Therefore the processes become labour intensive and time consuming. Furthermore, the process is very dependent on the user skills and the reproducibility of results is very difficult to achieve.

ii) *The use of toxic organic solvents:* The use of toxic solvents in technologies based in the casting of polymeric solutions may affect the biocompatibility of the resulting scaffold due to retention of harmful residues.

iii) *Use of porogens:* the methods based on porogen leaching are usually limited to the production of thin scaffolds to facilitate the total removal of the porogens, which can have a cytotoxic effect on the contacting cells. Furthermore, it is difficult to avoid the agglomeration of the porogen particles leading to non-uniform porosity and pore size.

iv) *Geometric limitations:* several techniques use moulds or containers to cast scaffolds that only allow for obtaining thin membranes and/or basic and uniform scaffolds geometries.

Nevertheless, one of the major problems of the scaffolds produced by most of the methods currently available is their poor mechanical properties and/or inadequate porosity. Obviously, it is very difficult to optimise these two properties simultaneously, since the mechanical properties are usually decreased with increasing porosity. However these are very important aspects for the regeneration of hard tissues because scaffolds are required to provide structural support for the neo-tissues and porosity and interconnectivity of the structures is essential to allow for cell proliferation within constructs with significant sizes. Therefore, the search for better methods of producing porous scaffolds, so that physical and chemical properties can be simultaneously optimised, is still an important and challenging issue especially in hard tissue engineering research.

Table I.1. Some examples of scaffolds aimed at applications in bone tissue engineering, based on different biodegradable polymers which were developed in recent years using different processing methodologies.

Processing method	Used polymers	References*
Fiber bonding	Hyluronic acid	[171]
	SPCL	[105,135]
Electrospinning	PCL	[172]
Solvent casting-particle leaching	PLLA,PLGA	[3,61,158-160,173-175]
	PLGA/PEG	[176]
	SCA	[123]
Supercritical fluid technologies	PLGA	[177-179]
	PLG/PGA/PLLA	[179]
Melt based technologies	PLLA,PLGA,PGA	[3,158]
Injection molding and extrusion with blowing agents	SEVA-C, SCA	[100,121,123]
Compression moulding-particle leaching	SCA	[121,123]
High pressure CO_2	PLGA	[1,67,165]
	PLA	[180]
Freeze-drying	PLLA	[1,158]
	PLGA	[181]
	PLG	[182]
Aggregation of microparticles	PLA	[3,166,167,183]
	PLAGA	[3,166,167,183]
In-situ polymerization	PPF	[158,184,185]
Microwave processing	SEVA-C	[103]
Rapid prototyping		
FDM	PCL	[168-170]
3D printing	Blend of Corn starch, dextran and gelatin	[186]

* Selected from works published between 1997 and 2004

PGA: Poly(glycolic acid); PLA: poly(lactic acid); PLG: poly(D,L-lactide-*co*-glycolide); PEG: polyethylene-glycol; PE: polystyrene; SEVA-C: starch/ethylene vinyl alcohol blend; SCA: starch/cellulose acetate blend; SPCL: starch/ Poly (ε-caprolactone); PPF: poly(propylene fumarate); PCL: Poly(ε-caprolactone);

In the following sections two different scaffold processing methods will be described with more detail, namely melt molding and fiber bonding, as these were the methodologies that were used to obtain the starch-based scaffolds that were more extensively studied under the scope of this PhD thesis, namely the scaffolds that were selected for the *in vitro* tissue engineering studies. A more detailed description of other processing technologies may be found elsewhere.[120,155]

2.3.1. Fiber bonding

The most important advantageous features of scaffolds obtained by fiber bonding processes, i.e., fiber meshes (which consist of individual fibers either woven or knitted into three-dimensional patterns of variable pore size), are a large surface area for cell attachment and a rapid diffusion of nutrients which enhances cell survival and growth.[3,24,61,67,158] This, of course, results from a high interconnectivity among pores. A drawback of these scaffolds might be the difficulty in controlling accurately the porosity [3,24,67,158]. Several studies demonstrate that scaffolds obtained by fiber bonding processes, have adequate structure for use in tissue engineering strategies that use bioreactor cultures, probably because they provide highly interconnected porosity that enables to create hydrodynamic micro-environments with minimal diffusion constrains that closely resemble natural interstitial fluid conditions in vivo allowing to achieve large and well organized cell communities. On the contrary, most of the porous obtained with other methodologies exhibit lower interconnectivity which is very likely to generate complex fluid flow pathways thought the scaffolds and that does not allow for the distribution of cells throughout the whole construct. Fiber bonding methods include a great variety of processing methods that involve the knitting or physical bonding (by means of casting or compression procedures) of fibers prefabricated by wet or dry spinning from polymeric solutions or by melt spinning.

Polyglycolic acid (PGA) non-woven meshes have been widely used in tissue engineering studies particularly concerning applications in cartilage reconstruction.[11,65,163,164,187-190] These meshes are produced by extrusion of PGA into fibers with 13μm of diameter which are subsequently stretched and relaxated at high temperatures, crimped and cut, carded into lofty web and finally needled to for a non woven mesh. Theses meshes are commercially available and are produced by Albany International (Mansfield, MA, USA).[11]

However, in spite of their wide use as scaffolds in tissue engineering, some authors believe that non-woven fibrous matrices require modification of their microstructure to organize cells in three-dimensional space with spatially balanced proliferation and differentiation in order to promote functional tissue development. In addition, the potential lack of structural stability for *in vivo* use has motivated the development of fiber bonding methods to modify this type of non-woven meshes into interconnected fiber networks with different shapes. For example, Mikos et al.,[3,24,61,67,158,191] developed a method of producing interconnected fiber networks by a fiber bonding technique that involves the casting of a PLLA solution over a non-woven mesh of PGA fibers. Solvent evaporation results in a composite material that consists of non-bonded PGA fibers embedded into a PLLA matrix. Fiber bonding occurs during a post treatment at a temperature above Tm of PGA. Finally the PLLA matrix is selectively dissolved in a non-solvent for PGA, and a network of bonded PGA fibers is released. However, stipulations concerning the choice of the solvent, immiscibility of the two polymers, and their relative melting temperatures restricts the general application of the technique to other polymers.[24,67,158] In addition, this method of fiber bonding does not address the problem of creating scaffolds with complex three-dimensional shapes, but it has proven successful for producing hollow tubes that have been proposed for use in intestine regeneration.[24,67,158]

A different method was developed by Li et al. [161], which is based on the thermal compression of non-woven polyethylene terephthalate (PET). It was found that a more uniformly distributed pore size resulted from thermal compression and the isotropic nature of non-woven fabrics was preserved because of the proportional reduction of the pore by compression. The thermally compressed fabric matrices with two different pore sizes (15 and 20 μm in pore radius) were used to culture human trophoblast ED27 and NIH 3T3 cells. It was found that cells cultured in the different pore-size PET matrices had different cell spatial organization and proliferation rates. The smaller pores in the matrix allowed for cells to spread better and proliferate faster, while cells in the larger pores tended to form large aggregates and had lower proliferation rate. According to the authors, this thermal compression technique can also be applied to other synthetic fibrous matrices, including biodegradable polymers used in tissue engineering, to modify the microstructure according to their viscoelastic properties.

In spite of a lot of work on fiber meshes has been focused on the use of synthetic polymers like the above mentioned PGA,[11,65,82,163,164,187-190] PLA[82] and polyethylene PET,[161] some natural origin polymers, such as hyluronic acid,[171,192] collagen[193,194] and blends of starch

with polycaprolactone (as it will be described in this thesis) have also been employed in the fabrication of fiber meshes for tissue engineering applications.
For example, a non-woven mesh based on the benzyl ester of hyaluronic acid (HYAFF 11) was investigated for application as a scaffolds material for the culture of human nasoseptal chondrocytes in tissue-engineering procedures of cartilage reconstruction [171]. The non-woven cell carrier allowed good viability and adhesion of the cells without any surface treatment with additional substances.[171] Another study, by Saldanha & Grande [193] has assessed properties of collagen as a scaffolding biomaterial for ligament replacements.

Fiber meshes may also be obtained in single step methods such as electrospinning. Electrospinning generates fibers with very small diameters (ranging from several microns down to 100 nm or less) which mimic the nanometer scales of the fibers that compose the extracellular matrix of native tissues.[195] In electrospinning, a polymeric solution or melt is injected with an electrical potential to create a charge imbalance and placed in proximity with a grounded target. At a critical voltage, a charge imbalance begins to overcome the surface tension of the polymer source, forming an electrical charged jet. The jet within the electrical field is directed toward the grounded target, during which time the solvent evaporates and fibers are formed. Electrospinning produces a single continuous filament that collects on the grounded target as a non-woven fabric.[196]
This technology has been used to process several natural and synthetic bioabsorbable polymers for biomedical applications [197] as for example, Poly(glycolic acid) [198], Poly(lactic acid),[199] Poly(D,L-lactide-co-glycolide),[200] Polycaprolactone,[201] poly(lactic acid) and polycaprolactone based blends,[202] Collagen,[196,203,204] Collagen-PEO blends[205] and elastin[202] poly(ethylene –co-vinyl alcohol).[195]

2.3.2. Melt molding

Melt moulding has been normally used in combination with porogen techniques or to produce a pre-shape of the final material, for example, to produce fibers that will be used in fiber bonding methods, as it was described above in the high pressure method that will be described further down in this section.
One example of application of this method consists in the use of a mixture of fine PLGA powder and gelatine microspheres that are loaded in a PTFE mould and then heated above the glass-transition temperature of the polymer.[3,24,67,158] The PLGA-gelatine

composite is subsequently removed from the mould and gelatine microspheres are leached out by selective dissolution in distilled deionised water. In this way, porous PLGA scaffolds with geometry identical to the shape of the mould can be produced. Polymeric scaffolds of various shapes can be produced by simply changing the mould geometry. This method also offers independent control of porosity and pore size [3,24,67,158] by varying the amount and size of microspheres used, respectively. In addition, it is possible to incorporate bioactive molecules in either polymer or gelatine microspheres for controlled drug delivery because this process does not utilises organic solvents and is carried out at relatively low temperatures for amorphous PLGA scaffolds. Besides the choice of gelatine, other leachable components may be used. This manufacturing technique may also be applied to PLLA or PGA. However, higher temperatures are required (above the polymer melting temperatures) because these polymers are semi crystalline and this excludes the potential for protein incorporation into these systems[3,24,67,158].

Melt based techniques have also been extensively studied to produce starch based scaffolds [100,121,123]. For example, it was developed a method based on compression moulding combined with salt leaching. In this method a starch based polymer is blended with leachable particles of different sizes, in sufficient amounts to provide a continuous phase of a polymer and a dispersed phase of leachable particles in the blend. The mixture is then compression molded into a desired shape and afterwards immersed in water to remove the salt particles, leaving an interconnected pore structure with controlled porosity and pore sizes. Starch based scaffolds have also been produced using melt moulding as a single method based on traditional melting technologies, such as injection moulding and extrusion with blowing agents. In these processes, the polymers are mixed with blowing agents, which are previously selected according to their decomposition temperatures, toxicity, etc, and then processed in an extruder or in an injection moulding machine. These methods allow for the productions of highly reproducible scaffolds with very complex 3D structures,[7,100,121,123] since it is possible to obtain scaffolds with the precise shape of the mould designed for specific applications. This type of technologies also offers the possibility of using a wide range of currently available equipments that can be used to produce, for example, bi-material scaffolds, i.e., scaffolds that may combine two different polymers and/or two different structures.

3. *IN VITRO* CULTURING OF CELL-SCAFFOLD CONSTRUCTS

3.1. Cell sources - bone marrow stromal cells

A further important consideration for the most widely studied tissue engineering approaches, which are based on the seeding and extended *in vitro* culturing of cells within the scaffold prior to implantation, is the cell source and the ability to control cell proliferation and differentiation. Primary cells derived from the patient's own healthy tissues (i.e., *autogenic cells*) could be the first obvious choice, since this avoids many of the problems associated with immune rejection of foreign tissues.[59,63] However these cells are not, in most cases, readily available in sufficient quantities for immediate use. By *in vitro* culture their number may be increased, but to reach a cell population necessary for a specific application may take from days to weeks,[59,63] mainly depending on cell type. Furthermore, the procedure of harvesting a tissue sample from the patient to isolate the necessary cells is always associated with significant morbidity in the site. Primary cells derived from normal donors of the same (i.e., *allogeneic cells*) or different species (i.e., *xenogeneic cells*) are, at least in concept, readily available in sufficient quantities due to the number of potential donors and to cryopreservation possibilities. However, in this case, rejection by the host's immune system and the possibility of diseases transmission, are serious risks to be considerer.[59,63] The use of cell lines can overcome some of these limitations, but these immortalized cells exhibit some of the properties of neoplastically transformed cells. Therefore, cells lines can be considered to be partially transformed cells with a predisposition to become fully neoplastic cells capable of forming tumors in the recipient. [59]

The recent identification of human embryonic stem cells[87,206-209] – cells that can give rise to essentially all cell types in the body, depending on the culturing conditions – offers probably the most exciting alternative source of cells for tissue engineering. However, researchers are still far from being able to control the differentiation of embryonic stem cells in culture.[208] In addition, the research on embryonic cells brings up a range ethical, political and legislative problems[87,206,207,209-211] that differ from country to country. This has driven researchers to actively investigate alternative stem cell sources, such as the adult stem cells [172,207,208,211-214]. These are undifferentiated cells that occur in differentiated tissues in theoretically the adult body but in fact from birth. They can renew themselves in the body, making identical copies of themselves for the lifetime of the organism, or become specialized to yield the cell types of the tissue of origin. Thus they are presently considered

as multipotent stem cells.[207,211-222] They are considered rare, often difficult to identify and purify, and when grown in culture, are difficult to maintain in the undifferentiated state. However there is a constant and significant effort to circumvent these problems in order to explore (in an advantageous form) all the possibilities arising from the use of adult stem cells. Sources of adult stem cells presently known include skin, brain, skeletal muscle, pancreas, fat, liver, the eye, dental pulp, the limit of the gastro-intestinal tract and maybe the most important, bone marrow and blood.[207,211-213,217,219,223-231]

The bone marrow is comprised of hematopoietic cells and adherent stromal cells of non-hematopoietic origin which together with the extracellular matrix provide a supportive scaffolding termed the bone microenvironment.[232,233] The cellular components of the marrow microenvironment include reticular endothelial cells, macrophages, adipocytes, fibroblasts and osteogenic precursor cells.[232,233]

Friedenstein and his coworkers[234-242] were the first to utilize *in vitro* cultured and transplantation in laboratory animals to characterize cells that compose the physical stroma of bone marrow. Due to the low amounts of extracellular matrix that are found in marrow, gentle mechanical disruption (usually by pipetting and passage through syringue needles of decreasing sizes) can readily dissociate stroma and hematopoietic cell into single-cell suspension. When these cells are plated at low density, bone marrow stromal cells rapidaly adhere and can be easily separated from the nonadherent hematopoetic cells by repeated washing.[243] Friedenstein et al.[234-242] has demonstrated that these culture-adherent cells present in the marrow stroma are capable of differentiating into bone and cartilage when placed into an appropriate environment *in vivo*. These experiments have lead to the hypothesis that stroma contains a unique population of stem cells (the mesenchymal stem cells) which are capable of differentiating along multiple cell lineages, including osteoblasts, chondrocytes and adipocytes, when placed in appropriate *in vitro* and *in vivo* environments.[233,235,244,245] Techniques and conditions that select for these cells in culture have been established for several animal species, including human. However, to date, the isolation of “pure” population of multipotent marrow stromal stem cells remains elusive.[243]

In humans, bone marrow can be harvested by aspiration from the superior iliac crest of the pelvis[214,244] and the associated morbidity is very low. Billions of marrow stromal cells can be generated from a limited amount of starting material, such as 1 ml of a bone marrow aspirate. Thus, these cells are rapidly adherent and capable of extended proliferation. From all the available sources of osteoblastic cells for bone tissue engineering, this is probably the only one that does not require an invasive surgical procedure or added time,

cost and risk for expand cells *in vitro*.[34] In addition, as cells can be harvested from the patient, the risk of disease transfer or immunological rejection is not an issue. The challenge lies in designing a microenvironment to provide the correct instructions to bring those cells to the desired functional state to create structurally organized tissues.[27,244,246]

As stated before, bone marrow stromal cells are a promising component for engineered bone tissues, but *in vitro* formation of bone-like tissue requires conditions that direct these multipotent cells towards osteoblastic maturation.[246] Several studies[25,26,55,232,247-261] have reported the *in vitro* differentiation of these cells, both from animal and human origin, along the osteoblastic lineage, usually revealed by their capacity of expressing and/ or synthesize bone matrix proteins, alkaline phosphatase and ability to form mineralized nodules. These studies[25,26,55,232,247-261] demonstrated that several bioactive agents are able to induce the osteogenic character of bone marrow stromal cells, namely serum, ascorbic acid, β-glycerophosphate and dexamethasone. Ascorbic acid (vitamin C) was found to be essential for collagen synthesis and secretion[255] while β-glycerophosphate provides an inorganic source of phosphate necessary for the occurrence of mineralization [255]. Dexamethasone has been extensively reported to stimulate osteogenic differentiation in bone marrow stromal cells form animal and human origin, demonstrated by morphological changes from an elongated to a more cuboidal cell shape and an increase in the expression and/or activity of alkaline phosphatase.[255] It has also been reported that this bioactive factor plays an essential role in the mineralization of marrow stromal cells.[255]
Other studies have focused on the influence of mechanical stimulus on the proliferation and osteogenic differentiation of these cells.[43,246,249,262,263]

3.2. *In vitro* culturing systems- bioreactors

Several recent studies demonstrate the importance of mimicking certain critical aspects of the native environment for the engineering of functional bone tissue substitutes. Therefore, besides the selection of the scaffold material and the cell source, it is necessary to develop more advanced procedures for culturing cells-scaffolds constructs in order to achieve microenvironments that encourage the cell and matrix organization to recapitulate the tissue´s natural structure and function, optimizing the *in vitro* culturing systems currently used. The most widely used culturing technique in tissue engineering studies is static culturing which is often characterized by non-homogenous cell distribution, confining the majority of the cells to the outer surfaces of the scaffold, which in turn results to an inhomogeneous distribution of the *in vitro* generated extracellular matrix.[58,264-266] In order

to overcome this limitation, several culturing systems which consist basically of growth chambers equipped with stirrers and sensors that regulate the appropriate amounts of nutrients, gases and waste products have been developed.[58,264-267] These systems, so-called bioreactors, may have different designs attempting to achieve one or more of the following objectives: i) maintain an uniform distribution of cells into the 3D scaffolds, ii) provide adequate levels of oxygen, nutrients, cytokines and growth factors iii) expose the cultured cells to mechanical stimuli. Furthermore, it is important to realize that engineered constructs of cells and scaffolds will be subjected to a complex biomechanical environment, potentially consisting of time-varying changes in stresses, strains, fluid pressure, fluid flow and cellular deformation.[42] These various physical factors, as it was suggested in previous sections, have the capability to influence the biological activity of normal tissues and therefore, may play an important role in the eventual success or failure of engineered tissues. In this sense, it is very important to characterize the diverse array of physical signals that cell may experience in vivo, as well as their biological response to such potential stimuli.[42] Experiments involving *in vitro* bioreactor culturing can also be designed to obtain such type of information, providing important insights into the long-term capability of engineered constructs to maintain the proper functionality.[264]

Bioreactors are also one of the focus of the development of a manufacturing technology for tissue engineered products, because they represent a chemically and mechanically controlled environment in which a tissue-like construct can be grown in reproducible conditions.[268] When the main purpose is to obtain engineered tissue-like substitutes, the type and the specific functional design characteristics of a bioreactor are determined by the dimensional and functional requirements of the tissue to be substituted/regenerated as well as by the cell-scaffold system used.

There are several types of bioreactors currently available, which can be grouped in three main types, namely the spinner flasks,[164,190,269] the rotating bioreactors[189,269-274] and the flow perfusion culture systems.[264,269,275-277]

3.2.1. Spinner flask

The spinner flask corresponds to one of the simplest biorreactor designs.[164,190,265,269,275] In these systems, the seeded scaffolds are attached to needles hanging from the cover of the flask and the mixing of the medium is maintained by a magnetic stir bar at the bottom of the flask. This mixing mechanism generates convective forces that enhance the nutrient

concentration gradients but only at the surface of the scaffolds. Nevertheless, these systems have shown to increase the cell number on cartilage constructs based on chondrocytes and fibrous polyglycolic acid scaffolds, while under static culture conditions cell growth rates are diffusionally limited due to increasing cell mass and decreasing effective construct porosity resulting from cartilage matrix regeneration.[188]
In another study [264], three-dimensional porous 75:25 poly(D,L-lactic-co-glycolic acid) biodegradable scaffolds were seeded with rat bone marrow cells (RBMCs) and cultured for 21 days under static conditions or in two model bioreactors (a spinner flask and a rotating wall vessel). The spinner flask culture demonstrated a 60% enhanced proliferation at the end of the first week when compared to static culture. Cell/polymer constructs cultured in the spinner flask had 2.4 times higher alkaline phosphatase (ALP) activity than constructs cultured under static conditions on day 14 and the total osteocalcin (OC) secretion in the spinner flask culture was 3.5 times higher than the static culture, with a peak OC secretion occurring on day 18. Furthermore, the spinner flask culture had the highest calcium content at day 14. On day 21, the calcium deposition in the spinner flask culture was 6.6 times higher than the static cultured constructs and over 30 times higher than the rotating wall vessel culture. Histological sections showed concentration of cells and mineralization at the exterior of the scaffolds at day 21. The accelerated proliferation and osteogenic differentiation of marrow cells and the localization of the enhanced mineralization on the external surface of the scaffolds, may be explained by the better mixing provided in the spinner flask, external to the outer surface of the scaffolds.

Spinner flasks can also be used as seeding systems, generating more homogenous and controlled cell distribution and density on the scaffolds. This was observed, for example in a study where, highly porous, fibrous polyglycolic acid scaffolds were seeded with bovine articular chondrocytes in spinner flasks. Essentially, all cells attached throughout the scaffold volume within 1 day. Mixing promoted the formation of 20-32-micron diameter cell aggregates that enhanced the kinetics of cell attachment without compromising the uniformity of cell distribution.[164]

3.2.2. Rotating wall vessel (RWV)

The rotating wall vessel (RWV) bioreactor was originally developed to protect delicate cell cultures from the high shear forces generated during the launch and landing of the space shuttle. Later on, when the device was tried for cell-line suspension cultures on the ground, cells were seen to aggregate and form larger structures resembling tissues. This

observation offered the exciting possibility that the bioreactor might be used to study the interactions of multiple cell types and their association with proliferation and cellular differentiation during the early steps of tissue formation.[270]
Nowadays, the rotating wall vessel bioreactor can have several different designs and can be used with either microcarrier suspensions or scaffolds.[189]
Basically, RWV bioreactors are horizontally rotated, fluid-filled culture vessels equipped with membrane diffusion gas exchange to optimize gas/oxygen supply. The initial rotational speed is adjusted so that the culture medium and the inoculum-individual cells, pre-aggregate cell constructs or tissue fragments-rotate synchronously with the vessel. As the cell aggregates grow in size, the rotational speed is increased to compensate for increased sedimentation rates. Under these conditions, at any given time, gravitational vectors are randomized and the shear stresses exerted by the fluid on the synchronously moving particles is minimized, thus establishing microgravity-like culturing conditions.

Two of the most well-known rotating wall vessel bioreactors designs are the High Aspect Ratio Vessel (HARV) and the Slow Lateral Turning Vessels (STLV).[274] The HARV has a flat membrane oxygenator at the rear of the chamber and the SLVT consists of a cylindrical growth chamber containing an inner co-rotating cylindrical with a gas exchange membrane.[274]
The oxygenation capacity of the HARV is higher than that of the SLTV and therefore, the HARV-type bioreactors are mostly used for cell types that require more oxygen per unit volume of culture medium while the SLTV-type bioreactors are suited for cells with low oxygen requirements. In a more advanced variant of the STLV, a fully automated computer controlled system continuously monitors flow through the rotating vessel, allowing for on-line monitoring of important parameters for cell development, such as pH, oxygen and glucose levels.[274]

The high aspect ratio vessel (HARV) systems have been used to investigate the formation of 3-D rat marrow stromal cell culture on microcarriers, specifically bioactive ceramic hollow microspheres, under conditions of simulated microgravity[271-273] these systems are aimed at applications as microcarriers for bone tissue engineering and as drug delivery systems.

In one of these studies, hollow ceramic microspheres coated with synthesized hydroxylapatite (HA) and sintered were developed and then placed in a rotating-wall vessel (RWV) bioreactor. The trajectory analysis revealed that the hollow microsphere remained suspended in the RWV bioreactor, and experienced a low shear stress

(approximately 0.6 dyn/cm^2). The cell culture studies performed using rat bone marrow stromal cells and osteosarcoma cells (ROS 17/2.8) showed that the cells attached to and formed 3-D aggregates with the hollow microspheres under the culture conditions provided by the RWV bioreactor. In additon, extracellular matrix was observed in the aggregates.[272]

In another study,[272] it was investigated the formation of 3-D rat marrow stromal cell culture on microcarriers and the expression of bone-related biochemical markers under conditions of simulated microgravity, using a high aspect ratio vessel (HARV) system. In addition, it was calculated the shear stresses imparted on the surface of microcarriers of different densities by the medium fluid in a HARV. Again, the examination of cellular morphology by scanning electron microscopy revealed the presence of three-dimensional multicellular aggregates consisting of multiple cell-covered microcarriers bridged together. Mineralization was observed in the aggregates. The expressions of alkaline phosphatase activity, collagen type I, and osteopontin were shown via the use of histochemical staining, immunolabeling, and confocal scanning electron microscopy. Using a numerical approach, it was found that at a given rotational speed and for a given culture medium, a larger density difference between the microcarrier and the culture medium (e.g., a modified bioactive glass particle) imparted a higher maximum shear stress on the microcarrier.

3.2.3. Flow perfusion bioreactor

The flow perfusion bioreactor is a bioreactor design that improves mass transfer at the interior of scaffolds.[275] The flow perfusion bioreactor uses a pump to perfuse medium continuously through the interconnected porous network of the seeded scaffold. The fluid path must be confined, so as to ensure the flow path is through the scaffold, rather than around the edges. The bioreactors that employ the latter flow path, i.e., exchanging medium in the chamber around the scaffold do not guarantee the exchange of medium within the interior of the scaffold and are termed “perfusion chambers”.[269] The perfusion bioreactor enhances the transport of nutrients because it allows the transport of medium through the interconnected pores of the scaffold. In addition, it offers a convenient way of providing mechanical stimulation to cells by means of fluid shear stress, which is particularly important in bone tissue engineering since bone cells are known to be stimulated by mechanical signals.[38,276] Furthermore, the magnitude of the shear stresses experienced by the cells can be varied by adjusting the flow rates through the systems. However, the local shear stresses experienced by individual cells are also dependent on the scaffold microarchitecture.[275]

Due to its characteristics, the flow perfusion bioreactor may facilitate the in-vitro development of tissue-like constructs for the regeneration of larger tissue defects. In addition, this culturing system also provides a valuable tool for *in vitro* investigation on biological mechanisms associated to bone growth and regeneration. In fact, the true biological environment of a bone cell derives from a dynamic interaction between responsively active cells experiencing mechanical forces and a continuously changing 3D matrix architecture, which can be simulated, obviously to a limited extent, in this type of bioreactor.

Several studies[276-278] have been carried out aiming at studying the differentiation and proliferation patterns of marrow stromal cells cultured in 3D titanium meshes under flow perfusion conditions. These studies demonstrated that under flow conditions (at different flow rates), mineralized matrix production was dramatically increased over statically cultured constructs with the total calcium content of the cultured scaffolds increasing with increasing flow rate. Flow perfusion induced *de novo* tissue modelling with the formation of pore-like structures in the scaffolds and enhanced the distribution of cells and matrix throughout the scaffolds. These results report on the long-term effects of fluid flow on primary differentiating osteoblasts and indicate that fluid flow has far-reaching effects on osteoblasts differentiation and phenotypic expression *in vitro.*[276] Further studies, using the same type of bioreactor, investigated the direct involvement of fluid shear stresses on the osteoblastic differentiation of marrow stromal cells. For this purpose, rat bone marrow stromal cells were seeded in 3D porous titanium fiber mesh scaffolds and cultured for 16 days in a flow perfusion bioreactor with perfusing culture media of different viscosities while maintaining the fluid flow rate constant. This methodology allowed exposure of the cultured cells to increasing levels of mechanical stimulation, in the form of fluid shear stress, whereas chemotransport conditions for nutrient delivery and waste removal remained essentially constant. Under similar chemotransport for the cultured cells in the 3D porous scaffolds, increasing fluid shear forces led to increased mineral deposition, suggesting that the mechanical stimulation provided by fluid shear forces in 3D flow perfusion culture can indeed enhance the expression of the osteoblastic phenotype. Increased fluid shear forces also resulted in the generation of a better spatially distributed extracellular matrix inside the porosity of the 3D titanium fiber mesh scaffolds. The combined effect of fluid shear forces on the mineralized extracellular matrix production and distribution emphasizes the importance of mechanosensation on osteoblastic cell function in a 3D environment.[278]

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Chapter II

MATERIALS & METHODS

Chapter II

MATERIALS & METHODS

1 - SCAFFOLDS DEVELOPMENT

1.1. Biodegradable polymers

The developed scaffolds were based in several biodegradable blends of corn starch with different synthetic polymers, namely:

- a blend of starch with ethylene vinyl alchool (50/50 wt %) - SEVA-C
- a blend of starch with poly(ε-caprolactone) (30/70 wt%) - SPCL
- a blend of starch with cellulose acetate (50/50 wt %) - SCA
- a blend of starch with polylatic acid (50/50 wt %) - SPLA

All these blends were obtained from Novamont, Novara, Italy.

1.2. Scaffolds obtained by extrusion and injection moulding with blowing agents

The polymeric materials were mixed in a rotating drum with one of the blowing agents described bellow, in amounts from 1% to 15% prior to processing by injection moulding or extrusion. In the injection moulding process it was used a Krauss Maffei KM60-120A injection moulding machine with a mould which was particularly designed for this application (developed at the Dept of Polymers Engineering, Univ. Minho) in order to allow maximal expansion and therefore enhance the formation of pores within the polymer melt. In the extrusion process, it was used a Carvex twin-screw extruder with a die diameter of 12 mm.

1.2.1. Blowing agents

The blowing agents have been carefully selected,[1-3] considering its decomposition temperatures and the melting temperatures of the polymer, but also considering the final application of the materials being developed since there has to a be special attention regarding to its composition and to the products released upon its decomposition, which can not be toxic.

This blowing agents used are mainly composed of carboxylic acids which react by heating, releasing CO_2 and water, forming the pores within the polymer melt.

The selection of suitable blowing agents as well as optimization of the processing conditions and cytotoxicity studies, has been a subject of previous studies.[1-3]
The blowing agents (BA) that were used to obtain the scaffolds studied in this thesis were:

- Hostatron, System P9947, Hoechst, Germany
- Hydrocerol BIH 70 and Hydrocerol BIH 40, Clariant, Germany.
- Celogen 780, Uniroyal Chemical, Germany

1.2.2. Processing conditions

The tables II.1 to II.4 present the optimised extrusion and injection moulding processing conditions that were used to obtain the scaffolds which were studied in this thesis.

Table II.1. Processing conditions used to obtain scaffolds based on SCA by extrusion with different blowing agents.

Blowing agent		**Processing Parameters**		
Type	**Amount (wt %)**	**Temperature profile:**	**Die Temperature (ºC)**	**Screw speed (rpm)**
Hostatron 9947	10%	160 - 165 - 170ºC	175	25
	15%			
Hostatron 9947 + 1% glycerol	10%			
BIH 40	2%	150 - 155 - 160ºC	165	17-18
	3%			
BIH 70	2.5			

Table II.2. Processing conditions used to obtain scaffolds based on SEVA-C by extrusion with a blowing agent

Blowing agent		**Processing Parameters**		
Type	**Amount (wt %)**	**Temperature profile:**	**Die Temperature (ºC)**	**Screw speed (rpm)**
BIH 40	**1%**	145 - 150 - 155ºC	160	24
	2%	155 -160 - 165 ºC	165	17
	3%	160 - 165 - 170ºC	170	17

Table II.3. Processing conditions used to obtain scaffolds based on SPLA by extrusion with a blowing agent

Blowing agent		Processing Parameters		
Type	**Amount (wt %)**	**Temperature profile:**	**Die Temperature (ºC)**	**Screw speed (rpm)**
BIH 40	**1%**	185 - 190 - 195ºC	200	20

Table II.4. Processing conditions used to obtain the scaffolds based on SCA by injection moulding with a blowing agent.

Material		Processing Parameters			
		Temperature profile: 140-160-175-180ºC			
Polymeric blend	**Blowing agent**	**Injection Pressure (MPa)**	**Holding Pressure (MPa)**	**Cooling Time (s)**	**Mold Temp. (ºC)**
SCA	1,5% CELOGEN 780	12,5	8	15	30

1.3. Compression moulding - particle leaching

The compression moulding and particle leaching was thought to overcome one of the main limitations of the methods based in the injection moulding and extrusion with blowing agents that is the difficulty of controlling the pore size and the porosity of the scaffolds. The compression moulding and particle leaching enables to control the percentage of porosity and the pore size by simply selecting the appropriate amount and size of the leachable particles used.

The compression moulding and particle leaching method was based on blending together a starch based polymer (in the powder form) and leachable particles (in this case, sodium chloride particles - NaCl, Merck) of different sizes (from 150 to 300μm), in sufficient amounts to provide a continuous phase of a polymer and a dispersed phase of leachable particles in the blend. The mixture of the polymer and leachable particles was then compression moulded in a press Moore, using a mould specially designed for this purpose. This mould produced discs with 6 cm of diameter and approximately 1 cm of height, which were subsequently cut into smaller samples according to the experience to be performed.

The resultant samples were then immersed in distilled water (to remove the leachable particles), for about 5 days, changing the water daily. Table III.5 presents the optimised conditions used to obtain scaffolds by the compression moulding-particle leaching method, based on SCA with 50 and 65% (wt%) of leachable particles .

Table III.5. Processing conditions used in the compression moulding of SCA and SPLA with different amounts of salt particles.

Material	Temperature (ºC)	Holding Time (min)
SCA + 50% salt	170	10-15
SCA + 65% salt		
SPLA + 65% salt	200	12-15

1.4. Solvent-casting/particulate-leaching

The solvent casting and particle leaching method has been widely used for producing scaffolds for tissue engineering. The procedure studied in this work was therefore based on those found in the literature[4-13]. However, there was a significant effort to overcome the disadvantages presented by the use of this technique as reported in the literature.[4-13] For example, in most cases, highly toxic solvents are used, which may leave residues in the final samples. Furthermore, in many cases, using this method, it was only possible to produce thin membranes, and/or materials with rather low mechanical properties.

The starch based polymers used to obtain scaffolds by solvent casting, in this case SCA and SEVA-C, were grounded in a high-speed milling (RETSCH) and then dissolved in acetic acid (Merck, Germany) (SCA) and dimethylsulfoxide (Aldrich, Germany), respectively. It was obtained a very viscous solution by mixing approximately 12 g of the polymer powder with 20 mL of the solvent and then it was added the leachable particles (NaCl). Usually, after preparing the polymeric solution it is necessary to allow the evaporation of the excess solvent (for about 1 day) before adding the salt particles, in order to obtain a better dispersion of the particles within the polymeric solution. The amount and size of the used salt particles determined the amount and size of the pores in final sample. In general, it was added a salt weight fraction of 60 to 70% (based on the total mass of polymer and salt). The size of the particles used ranged from 50 to 1000μm. The mixture of the polymeric solution with the salt particles was then poured into a mould

(a glass petri-dish of 3cm diameter) and placed in an oven at 37ºC in order to allow a progressive evaporation of the solvent, for about 4 to 5 days. Finally, when the samples were completely solidified, they were cut into smaller samples (according to the tests to be performed) and immersed in distilled water during several days (about one week), for leaching of the salt particles, being afterwards dried.

1.5. In-situ polymerization

This innovative so-called in situ polymerization process was based on a polymerization process developed in our group in order to obtain materials to be used as bone cements or hydrogels[14-16]. The innovation introduced by this method of obtaining scaffolds for tissue engineering lies in the fact that it is possible to produce the scaffold in-situ, i.e., it might be possible to inject the scaffold directly into the defect to treat , which can, therefore, take immediately the shape of the defect.

Materials produced by this method are not totally degradable, but they might be very useful in situations where it is necessary high mechanical properties and/or in situations where the defect or trauma that is necessary to treat is of difficult access, avoiding highly invasive surgery techniques.

These materials were prepared by adding the liquid phase, constituted by the acrylic monomers (from acrylic acid, Merck), and 1% (w/w) of DMOH, to the solid phase, which consisted of SEVA-C powder and 2% (wt/wt) of BPO. N-dimethylaminobenzyl alcohol (DMOH), was used as the activator of the initiation process, after being synthesised by selective reduction of 4-dimethylaminobenzaldehyde with sodium borohydride in alkaline medium[17]. Benzoic peroxide (BPO, Merck), was used as the radical initiator, after purification by fractional recrystallization from ethanol and subsequently vacuum dried (m.p. 104 ºC).

The leachable NaCl particles (sized between 150 to 300 μm) were added to the liquid or to the solid phase. The solid and the liquid phases are then mixed together with a 10% of water with respect to the total weight and poured in a dough state in poly (tetrafluoroethylene) (PTFE) moulds until complete polymerisation take place. A total amount of 4g were used to prepare each sample of 4 cm of diameter.

After curing time, about 5 minutes, moulds are placed into the oven at 60ºC overnight to ensure a complete polymerization and then vacuum dried until constant weight was attained. Finally the samples were immersed in water to leach out the salt particles.

1.6. Scaffolds obtained by fiber bonding processes

Scaffolds based on SPCL (a 30/70 wt% blend of starch with poly(ε-caprolactone)) were prepared by a fiber bonding process consisting of cutting and sintering fibers with a diameter of about 180μm, obtained by melt-spinning. The fibers used were previouslly produced by melt spinning by Novamont (Novara, Italy). The typical processing conditions for the extrusion of SPCL fiber are presented in table III.6.

Table III.6. Processing conditions used to obtain SPCL fibers by extrusion

Polymer	**Processing Parameters**		
SPCL	**Temperature profile**	**Die Temperature**	**Geometry of the die**
	100-130-140ºC	150 ºC	D=0.5-0.7, L/D=2-3

The procedure for obtaining the fiber meshes is based on a method developed by Novamont, Italy: The SPCL fiber meshes were cut into 1-3 cm pieces and then washed with distilled water to remove plasticizers. A selected amount of fibers was placed in a 10 ml glass beaker; for the studies described in this thesis, we have prepared scaffolds with two different porosities, namely 50 and 75%; the different porosity of the fiber meshes was obtained using different amounts of fibers.

A glass cylinder (which runs within the beaker), is let drop (by gravity) several times to compact the fibres. The cylinder is then removed and the beaker is placed in an oven at 150ºC during 5 minutes. Immediately after removing the beaker from the oven, the glass cylinder is dropped once to compress the heated fibers. The beaker with the fibers is then transferred to a freezer at –15ºC for about 10 minutes.

2. CHARACTERIZATION OF THE POROUS STRUCTURES

2.1. Morphological characterization

The porous structure of the materials developed was characterised by scanning electron microscopy (SEM), in a Leica Cambridge S360. All the samples were previously gold coated in a Sputter Jeol JFC 1100 equipment.
The SEM analysis allowed to evaluate the morphology of the pores, their size and distribution and also the interconnectivity between these pores.

The porosity of the scaffolds was determined by microcomputerized tomography (μCT) (ScanCo Medical μCT 80, Bassersdorf, Switzerland) at a resolution of 10 μm, and using at least 3 samples per group (of different porosity).

Estimations of the porosity of the scaffolds were also obtained from calculations of the apparent density of scaffolds based on SEVA-C and SCA, using the equations II.3 and II.4:

$\rho^* = m/V$ — ρ^*= apparent density; m = weight of the sample; V= volume of the sample — **(Equation II.1)**

$$\varepsilon = \frac{1 - \rho^*}{\rho} \times 100\%$$

ε = porosity; ρ = density of the polymer (for SEVA-C, ρ=1.26; for SCA, ρ=1.28) — **(Equation II.2)**

2.2. Degradation behaviour

The degradation behaviour was assessed after several pre-fixed ageing periods (0,3, 7, 14, 30, 60 and 90 days), in an isotonic saline solution (NaCl 0.154 M). At the end of each degradation period, the samples (n=4) were removed from the solution, rinsed with distilled water and weighted, to determine the water uptake, according to the equation II.1 shown bellow; one batch of samples was then dried up to exhaustion (6 days at 60ºC) in order to determine the dry weight loss, using equation II.2 described bellow; the other batch of samples was dried in a controlled environment of 23ºC and 55% RH, to be tensile tested in

order to evaluate the changes in the mechanical properties as a function of the degradation time.

$$\% \text{ water uptake} = \frac{\text{final wet weight - initial weight}}{\text{initial weight}} \times 100\% \qquad \textbf{(Equation II.3)}$$

$$\% \text{ weight loss} = \frac{\text{initial weight - final dry weight}}{\text{initial weight}} \times 100\% \qquad \textbf{(Equation II.4)}$$

2.3. Mechanical properties - compression tests

The developed materials were mechanically tested on compression experiments in an Instron 4505 universal mechanical testing machine, using a load cell of 50 kN.
Compression testing was carried out at a crosshead speed of 2 mm/min (4.7×10^{-5} m/s), until fracture or until obtaining a maximum reduction in samples height of 60%. At least six samples of each type were tested. As the different processing methods produced scaffolds of different geometries, the samples tested have also different geometries and dimensions, as shown in Table II.7.

Table II.7. Typical geometry (and dimensions) of the several scaffold samples (obtained by different processing methodologies) tested on compression.

Processing method	Geometry of sample	Dimensions
Extrusion with blowing agent	Cylindrical	Height: 15 mm Diameter: 12mm
Injection moulding with blowing agent	Rectangular	Height: 9.5 mm Length: 16mm Width:16 mm
Compression moulding + particle leaching	Rectangular	Height: 8.5 mm Length: 24mm Width:18mm
Solvent casting + particle leaching	Rectangular	Height: 8.5 mm Length: 16 mm Width: 14 mm

3. CULTURING OF CELLS-SCAFFOLDS CONSTRUCTS

3.1. Samples preparation

Several starch-based scaffolds were developed, using different starch based blends and distinct processing methodologies. For the cell culturing experiments, two types of scaffolds were selected, namely the scaffolds obtained by extrusion of SEVA-C with a blowing agent and fiber-mesh scaffolds based on SPCL.

Several studies[18-22] refs indicated that fiber-mesh scaffolds might have more adequate structures for use in tissue engineering strategies that use bioreactor cultures, probably because they provide highly interconnected porosity. On the contrary, most of the porous obtained with other methodologies exhibit lower interconnectivity which is very likely to generate complex fluid flow pathways thought the scaffolds. Nevertheless, SEVA-C scaffolds was thought to provide evidence of the influence of scaffold architecture, namely of their porosity and pore interconnectivity, on the development of functional bone tissue engineered constructs.

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All samples used in cell culture experiments were previously cut into discs of approximately 8 mm diameter and 1.5-2 mm high and EtO sterilized at Pronefro (Maia, Portugal) or at the Dept of Bioengineering (Rice Univ., Houston, USA). Typical conditions include a working temperature of 45ºC, a moisture level of 50%, a cycle time of 14 hours and a chamber pressure of 50kPa.

3.2. Isolation and expansion of rat bone marrow cells

Rat bone marrow (RBM) cells were isolated and cultured using the method described by Maniatopoulos[8]. The animals, (Wistar rats from Harlan, USA) were euthanized using $CO_{2,}$ under isofluorane anaesthesia. The femurs and tibias were removed and briefly Immersed in ethanol (70%) and then in culture medium (without osteogenic supplements) containing a higher percentage of antibiotics. The epiphyses are cut off and the diaphyses flushed with 5 ml of complete medium, removing the bone marrow to 50 ml tubes. The mixture (bone marrow + medium) was ressuspended with a plastic Pasteur pipette (see Figure II.1) and then transferred (5 mL) to 75cm2 culture flask previously filled with 10mL of complete culture medium. After 24 hours, the medium was replaced very carefully, avoiding movement as much as possible and then changed each 2 days until day 6. The

hematopoetic cells present in the marrow cell preparations usually do not attach to culture flasks and are removed during feeding in the primary cultures[23,24] . This way they are easily separated from the bone marrow stromal cells that rapidly adhere to culture flasks.

The medium used in all experiment was α-MEM (Minimal Essential Medium Eagle; MEM alpha modification-M0644, Sigma, USA), supplemented with 10 % FCS (foetal calf serum, Gemini, USA), 50 μg/ml ascorbic acid (Sigma, Chemical Co., St.Louis, MO, USA), 50 μg/ml gentamycin, 100μg/ml ampicillin, 3 μg/ml fungizone, 10 mM Na β-gycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma). Several studies[23,25-42] have demonstrated the ability of culture medium supplemented with these osteogenic supplents, namely ascorbic acid, β-glycerophosphate and dexamethasone, to induce the osteogenic character of bone marrow stromal cells. Ascorbic acid (vitamin C) was found to be essential for collagen synthesis and secretion[35] while β-glycerophosphate provides an inorganic source of phosphate necessary for the occurrence of mineralization[35]. Dexamethasone has been reported[35] to stimulate osteogenic differentiation in bone marrow stromal cells form animal and human origin, demonstrated by morphological changes from an elongated to a more cuboidal cell shape and an increase in the expression and/or activity of alkaline phosphatase. It has also been reported that this bioactive factor plays an essential role in the mineralization of marrow stromal cells[35].
Cells were cultured in a humidified atmosphere of 5 % CO_2 at 37°C during 6 days.

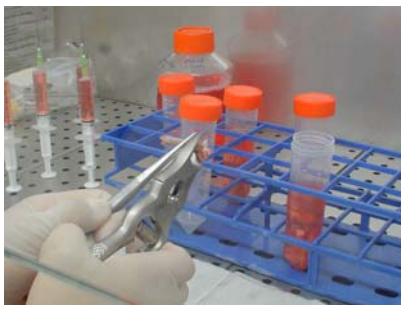
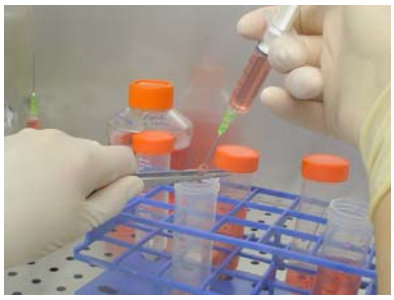
a)  b)  c) 

Figure II.1. Some sequential steps of the procedure for isolation of rat bone marrow cells: a) Cutting off the epiphyses of the rat femur b) flushing the bone marrow with 5 ml of culture medium and transfer to 50 ml tubes. b) ressuspending the bone marrow.

3.3. Cell seeding on starch based scaffolds

3.3.1. Pre-wetting of the samples

The samples were immersed in 30 ml of serum free medium in 50 ml tubes and the air removed by means of a 30 ml syringue with a 18 g needle. The scaffolds were left in the serum free medium overnight to allow swelling.

3.3.2. Seeding

After 6 days of primary culture, cells were detached using trypsin/ EDTA (0.25% w/v trypsin / 0.02% EDTA, Sigma). The cells were concentrated by centrifugation at 1500 rpm for 5 min and resuspended in media. Subsequently, the scaffolds (n=6 for flow and n=6 for static culture, for each culture period), were inserted into the cassettes (see figure II.1) which were placed in a 6-well plates and seeded with 300μl of a cells suspension containing $5x10^5$ cells. After 2 hrs of attachment, 10 ml of complete medium was added to the each well. Seeded scaffolds were incubated for further attachment overnight. The following day seeded scaffolds were placed into fresh 6-well plates for static conditions or into the flow perfusion system for culturing during the selected time periods.

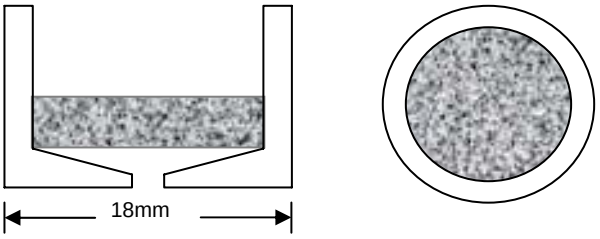


Figure II.2. Schematic representation of the cassette, containing the scaffold, a) side and b) top view).

3.4. Cell culturing: the flow perfusion culture system

The Flow Perfusion Culture System that was used in this thesis was developed in the Department of Bioengineering of Rice University[43]. This type of bioreactors uses a peristaltic pump to perfuse medium continuously through the interconnected porous network of the seeded scaffold. The fluid path is confined to ensure the flow path is through the scaffold and not around the edges. In this manner, medium is delivered throughout each scaffold, enhancing delivery of nutrients and providing mechanical

stimulation to the seeded cells by way of fluid shear stresses.

This flow perfusion bioreactor was machined from Plexiglas and is mainly composed of a main part with of flow chambers, a peristaltic pump and two medium reservoirs. Each flow chamber contains a cassette in which the scaffold is press-fit. Each cassette is then sealed with two o-rings to ensure the flow path goes only through the scaffold. Silicon tubing then connects each flow chamber with a multichannel peristaltic pump and a medium reservoir. Each chamber is on its own independent pumping circuit, but all pumps draw media from a common reservoir. For these experiments, culture media was pumped continuously at a flow rate of 0.3ml/min or 1 ml/min through the cell/scaffold construct cassette/housing unit and recirculated back to the reservoir. These flow rates were selected with basis on previous experiments performed using titanium fiber-meshes[19]. In fact, these are several reports[19,21] on studies using these non-degradable scaffolds and this flow perfusion bioreactor, but this thesis reports on the first studies using biodegradable scaffolds, as part of a tissue engineering therapy which aims at substituting temporary bone defects while the tissue is being regenerated.

The total volume of culture medium in the flow system was 210mL and in static cultures was 10mL per well. In both cases, the whole volume of medium was changed every 3 days. The entire culture system was sterilized by ethylene oxide sterilization and maintained at 37°C with a 5% CO_2 environment. The silicon tubing was replaced in every new experiment.

Table II.8. Experimental conditions used in bioreactor cultures.

Constant conditions		**Variable conditions**	
Total Volume of medium:	210 ml	**Flow rate:**	0.3 ml/mim 1ml/mim
Frequency of medium change (total volume change):	Each 3 days	**Culture periods:**	3 days 7 days 10 days 15 days
Total number of samples in the bioreactor:	6 samples		

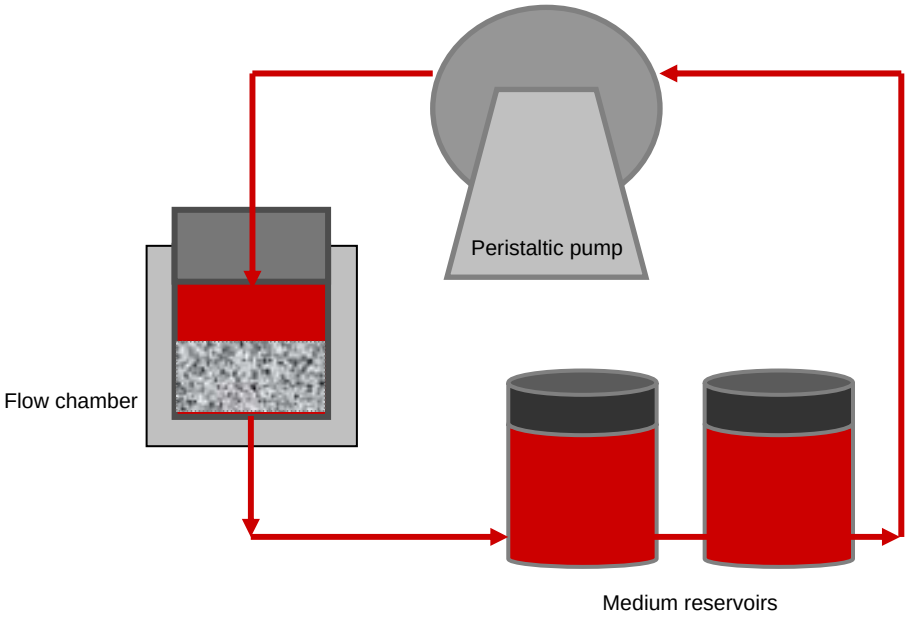


Figure II.3. Schematic representation of the flow circuit diagram of the flow perfusion system of only one of the six chambers. Adapted from Brancroft et al.[43]

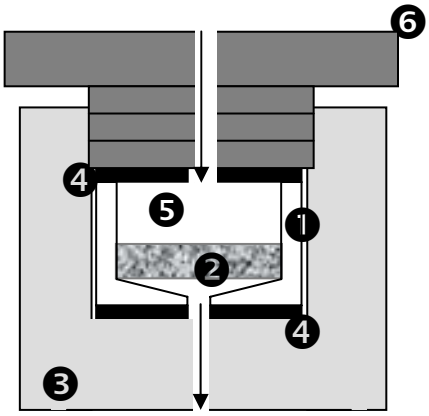


Figure II.4. Representation of one flow chamber in the main part of the flow perfusion bioreactor: the cassette (1) with the scaffold (2) is inserted in the flow chamber (3). The O-rings (4) placed above and bellow the cassette prevent linkage and ensure the flow path (indicated by the arrows) goes only through the seeded scaffold. A screw top (6) closes the flow chamber. Adapted from Brancroft et al.[43]

4. CHARACTERIZATION OF THE CULTURED CELL-SCAFFOLDS CONSTRUCTS

4.1. Biochemical analysis

For DNA, Calcium and ALP analysis. at the end of each culturing period selected, the cultured cell-scaffold constructs were rinsed with PBS and stored at -70°C in 10ml tubes containing 1.4 ml of milliQ water.

4.1.1. Determination of cellularity of scaffolds

The DNA content of each scaffold was measured in order to evaluate the cell proliferation using the PicoGreen dye and buffers contained in the Molecular Probes PicoGreen dsDNA Quantification Kit (P-7589). This assay measures the fluorescence produced when PicoGreen dye is excited by UV light while bound to dsDNA. The protocol used is described below. First the homogenates stored at –70ºC were allowed to thaw at room temperature and sonicated for about 15 minutes. Aliquots of 43 μl of each sample were then placed into individual wells of a 96-well plate. Standards ranging from 0 – 6 μg/ml were made from dilutions of a standard solution of calf thymus DNA (100μg/ml). Aliquots of 43 μl of these standards were placed into other wells. Tris-EDTA buffer was prepared according to the manufacturer's instructions using reagents provided in the kit and 107 μl added to each of the sample and standard wells. The PicoGreen dye solution was also prepared according to the manufacturer's instructions using reagents provided in the kit and 150 μL added to each of the sample and standard wells. The 96-wells plate was then incubated in the dark for 10 minutes at room temperature and the fluorescence then measured on a fluorometer using an emission wavelength of 490 nm and an absorbance wavelength of 520 nm. From these dsDNA determinations the cellularity of each scaffold was estimated using a previously determined[19] conversion factor of 2.7 pg dsDNA/cell and the results presented as number of cells per scaffold.

4.1.2. Determination of alkaline phosphatase activity of cells on scaffolds

The alkaline phosphatase activity of each scaffold was assayed as a measure of the osteoblastic expression of the cells present. The alkaline phosphatase activity was measured using Sigma Diagnostic Kit #104. This columetric endpoint assay measures the conversion of the colorless substrate p-Nitrophenol phosphate by the enzyme Alkaline Phosphatase to the yellow product p-Nitrophenol. The method is described in detail in the

instructions provided with the kit. Briefly, the scaffolds were rinsed twice with calcium-free phosphate buffered saline and then homogenized in 1.4 ml of filtered ddH_20. The homogenates (frozen overnight at -70°C) were allowed to thaw at room temperature and then sonicated for about 15 minutes. Aliquots of 80 μl were then taken from each sample and placed into microplate wells on a 96-well plate. Standards in concentrations ranging from 0 – 250 μM were prepared from dilutions of a standard solution of p-Nitrophenol (10μmol/ml) provided in the kit. Aliquots of 80 μl of these standards were added to the same 96-well plate. The Working Alkaline Buffer Solution was prepared according to the manufacturer's instructions using reagents provided in the kit and 20 μl of this buffer was added to each of the sample and standard wells. The Substrate Solution was also prepared according to the manufacturer's directions using reagents provided in the kit and 100 μl of this solution will be added to each of the sample and standard wells. The microplate was then incubated for 1 hour at 37°C in a cell culture incubator. After this, aliquots of 100 μl from a Stop Solution consisting of 0.3 M NaOH in ddH_20 were added each of the sample and standard wells. Finally, the absorbance of each well at 405 nm was measured on a plate reader. The results obtained were normalized to the number of cells in each scaffold and presented as pmol per hour per scaffold.

4.1.3. <u>Determination of calcium content of scaffolds</u>

The calcium content of each scaffold was assayed in order to quantify the amount of mineralized matrix present. The scaffolds were incubated overnight in 1N acetic acid to dissolve the calcium in the scaffolds. The calcium content was then measured using Sigma Diagnostic Kit #587. This columetric endpoint assay measures the amount of purple-colored calcium-cresolphthalein complexone complex formed when cresolphthalein complexone binds to free calcium in an alkaline solution. The method is described in detail in the instructions provided with the kit. Briefly, aliquots of 10 μl were taken from each sample and placed into microplate wells on a 96-well plate. A stock solution of 1 mg/ml $CaCl_2$ was prepared and standards in concentrations ranging from 0-300 μg/ml were prepared from dilutions of this stock solution. Aliquots of 10 μl of these standards were added to the same 96-well plate. The Assay Working Solution was prepared by mixing equal parts of the Calcium Binding Reagent and Calcium Buffer Reagent provided in the kit. Aliquots of 300 μL of the Assay Working Solution were added to each well containing sample or standard aliquots in the 96-well plate. The 96-well plate was then incubated for 10 minutes at room temperature and then the absorbance of each well at 575 nm

measured on a plate reader. The results were expressed as mg of Ca^{2+} equivalents per scaffold.

4.1.4. Statistics

Several samples were used for each measurement (n=3 to 5) and all the values are presented as averages ± the respective standard deviations. Multiple pairwise comparisons have been performed using the Turkey-Kramer method with a significance level of 95%.

4.2. Histology and imaging of tetracycline fluorescence

The cultured samples were rinsed with PBS, fixed in formalin for 1 week at 4ºC, rinsed with water and embedded in frozen tissue embedding media (HistoPrep™, FisherDiagnostic). Sections of about 30 μm were obtained using a cryotome (Microm 505) and stained with hematoxylin and eosin for general histological evaluation. For visualization of mineralized tissue, additional sections were exposed to a 5% silver nitrate solution under UV light for 25 minutes and counter stained with a safranin-O solution (0.5%). Mineral deposition was also observed after adding tetracycline-HCl (10μg/ml) to the culture media. Tetracycline is known as a fluorochrome-labeling agent for bone tissue as it accumulates at bone forming sites and fluoresces when activated with fluorescent light. All the histological sections were observed in a light microscope Nixon E600 equipped with a Sony DXC-950P CCD camera and with a fluorescence lamp (for imaging of tetracycline fluoroscesce).

4.3. Immunohistochemistry analysis

4.3.1. Samples processing

At the end of each culturing period, the cell-mesh constructs were removed from the bioreactors, rinsed with a phosphate buffer saline solution (PBS, 0.01M, pH 7.4) and fixed in a 10% formalin solution (Sigma). The constructs were then rinsed with PBS, cut in halves, embedded in optimal freezing temperature (O.C.T.) compound (Tissue-Tek, USA) and frozen on dry ice. Serial sections (10 μm in thickness) were prepared and stored at –80ºC until staining.

4.3.2. Immunostaining procedure

The sections were immunostained using previously established protocols[44] using the avidin-biotin immunoperoxidase staining technique which is based on the ability of egg-white glycoprotein avidin to non-immunologically bind four molecules of the vitamin biotin [45]. This technique uses 3 main reagents: the first is a primary antibody specific for the antigen to be localized; the second is antimouse/antigoat polyclonal antibody (secondary) which is covalently linked to a molecule of biotin and the third is a complex of peroxidase conjugated with biotin and avidin. The free sites on the avidin molecule allow binding to the biotin on the second antibody. The peroxidase enzyme, and therefore the original antigen, is identified with an appropriate chromogen [45].

The experimental staining procedure can be briefly described as follows: the sections were first incubated with a hydrogen peroxide solution to block against endogenous peroxidase activity and then incubated with normal serum to block against random secondary antibody binding. Afterwards, the sections were incubated with primary antibody for the antigen of interest and then incubated with the avidin-biotin secondary antibody system and with 3,3'-diaminobenzidin developing reagent (DAB, Vector Laboratories). As a last step, the sections were counterstained with hematoxylin and mounted. The negative staining controls consisted of sections incubated with 0.01M PBS instead of the primary antibody. A group of samples received conventional hematoxilin and eosin staining.

4.3.3. Primary antibodies

For this study, the following antibodies were selected: anti-transforming growth factor-β1 (anti-TGF-β1, goat polyclonal antibody, sc-146-G), anti-platelet derived growth factor-A (anti-PDGF-A, mouse monoclonal antibody, sc-9974), anti-fibroblast growth factor-2 (anti-FGF-2, goat polyclonal antibody, sc-79-G), anti-vascular endothelial growth factor (anti-VEGF, mouse monoclonal antibody, sc-7269) and anti-bone morphogenetic protein-2 (anti-BMP-2, goat polyclonal antibody, sc-6895). All the antibodies were purchased from Santa Cruz Biotechnology (California, USA) and used at a concentration of 2μg per ml of 0.1 PBS. The diluted antibody solutions were used in the same day they were prepared. The mouse monoclonal antibodies were used in conjunction with an anti-mouse avidin-biotin complex ABC kit and the goat polyclonal antibodies were used in conjunction with an anti-goat ABC kit (both Vectastain Elite® ABC kit, Vector Laboratories).

4.4. Microscopy analysis

For analysis under scanning electron microscope and confocal microscope, the samples were fixed in a solution of 2.5% glutaraldehyde and for histology and histochemical analysis the samples were fixed in a 10% formalin solution.

4.4.1. Scanning electron microscopy

For SEM analysis the samples were fixed in a solution of 2.5% glutaraldehyde (in PBS), dehydrated in a gradient series of ethanol (35, 50, 70, 80, 90, 95 and 100%), dried with tetramethylsilane and sputter coated with gold. Samples were then observed in a Leica Cambridge S360 scanning electron microscope.

4.4.2. Confocal microscopy

In order to visualize cell distribuition within the scaffolds, the scaffolds cutured for 15 days (previously fixed with glutaraldyede) were cut in half (in order to visualize the interior cross section of the samples), rinsed with PBS and incubated with a picogreen dye (0.1%) for at least 15 minutes and then observed under a confocal microscope (Zeiss LSM Axiovert, Carl Zeiss, Germany).

Depth projections of the surface (up to 300μm) were also obtained and the images were pseudo-coloured to show depth as a function of colour.

4.4.3. Light Microscopy

All the histological sections were observed with an Eclipse E600 light microscope (Nikon, Melville, NY, USA) equipped with a CCD camera (Sony DXC-950P, NY, USA) and with a fluorescence lamp (for imaging of tetracycline fluoroscesce).

In the case of the sections immunohistochemically stained, the Image acquisition was performed as follows: for each stained section, 3 digital images were taken, corresponding to the opposite ends and center of the sample (see figure VII.1, chapter VII), at a magnification of x4.

4.5. Spectroscopy and diffraction

Diffraction or spectroscopy methods were used to analyse the mineral deposition on cell-scaffolds constructs cultured for 15 days under flow perfusion conditions. The use of these techniques aimed at complement the analysis by the biochemical assay (calcium deposition) and histological staining (Von Kossa) because the results arising from these analyses can be misinterpreted, as the matrix is known to uptake calcium independently of mineral deposition.[46]

4.5.1. Fourier transformed infrared spectroscopy with attenuated total reflectance (FTIR-ATR)

The composition of the cell-scaffolds constructs cultured for 15 days in the perfusion bioreactor was analyzed by Fourier Transformed Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR). The samples were fixed in a solution of 2.5% gluteraldehyde and dried at room temperature. All spectra were recorded using at least 64 scans and $2cm^{-1}$ resolution in a FTIR spectrophotometer (Perkin-Elmer 1600 Series, USA) with a single reflection ATR system (MKII Golden Gate™, Specac, UK). Results were compared to cell-free scaffolds, which were kept under the same conditions as the static cultures and received the same treatment before performing the analysis, in order to exclude the contributions from the culture medium.

4.5.2. Thin-film X-ray diffraction (TF-XRD)

Thin-film X-ray diffraction (TF-XRD, Philips X'Pert MPD, The Netherlands) was used to identify any mineral phase present in cell-scaffolds constructs cultured in the perfusion bioreactor for 15 days. As for the FTIR-ATR analysis, the samples were dried at room temperature after being fixed in a solution of 2.5% gluteraldehyde. The data collection was performed by the 2θ scan method with 1º as incident beam angle using Cu$K\alpha$ X-ray line and a scan speed of 0.05º/min in 2θ. Again, the results were compared to cell-free scaffolds, which were kept in the same conditions and submitted to same treatment as samples resulting from static cultures.

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Chapter III

ALTERNATIVE TISSUE ENGINEERING SCAFFOLDS BASED ON STARCH: PROCESSING METHODOLOGIES, MORPHOLOGY, DEGRADATION AND MECHANICAL PROPERTIES

Chapter III

Alternative Tissue Engineering Scaffolds Based on Starch: Processing Methodologies, Morphology, Degradation and Mechanical Properties*

Abstract

An ideal tissue engineering scaffold must be designed from a polymer with an adequate degradation rate. The processing technique must allow for the preparation of 3-D scaffolds with controlled porosity and adequate pore sizes, as well as tissue matching mechanical properties and an appropriate biological response.

This communication revises recent work that has been developed in our laboratories with the aim of producing 3-D polymeric structures (from starch based blends) with adequate properties to be used as scaffolds for bone tissue engineering applications. Several processing methodologies were originally developed and optimized. Some of these methodologies were based in conventional melt based processing routes, such as extrusion using blowing agents and compression moulding (combined with particulate leaching). Other developed technologies included solvent casting and particle leaching and an innovative in-situ polymerisation method.

By means of using the described methodologies it is possible to tailor the properties of the different scaffolds, namely their degradation, morphology and mechanical properties, for several applications in tissue engineering. Furthermore the processing methodologies (including the blowing agents used in the melt based technologies) described above do not affect the biocompatible behaviour of starch-based polymers. Therefore scaffolds obtained from these materials, by means of using one of the described methodologies may constitute an important alternative to the materials currently used in tissue engineering.

*** This chapter is based on the following publication:**

ME Gomes, JS Godinho, RL Reis, AM Cunha. *Alternative Tissue Engineering Scaffolds based on Starch: Processing Methodologies, Morphology, Degradation Behaviour and Mechanical Properties.* Materials Science and Engineering: C Biomimetic and Supramolecular Systems (2002) **20**:19-26

1. INTRODUCTION

The advent of tissue engineering has been motivated by the challenge of producing tissue substitutes that can restore the structural features and physiological functions of natural tissues *in-vivo* [1,2], circumventing the limitations of current therapies for tissue mal-functioning or tissue loss. Tissue engineering is an interdisciplinary science that combines the knowledge of distinct fields, from biology to materials science and engineering, to obtain hybrid materials to use in substitution medicine. In most cases, biocompatible, degradable polymers are utilised to induce surrounding tissue ingrowth or to serve as temporary scaffolds for transplanted cells to attach, grow and maintain differentiated functions[2-7], which then degrade, as the new tissue is formed.

These materials must comply with a large number of requirements. Besides the obvious demand of biocompatibility and biodegradability, they should have other properties such as the appropriate mechanical properties, to provide the correct stress environment for the neo tissue[1,5,8-14], the adequate degradation rate that assures that the strength of the scaffolds is retained until the newly grown tissue takes over the synthetic support[2,5,10,12-15], the adequate porosity and permeability in order to allow the ingress of cells and nutrients,[2,5,8,12-15] and the appropriate surface chemistry for enhanced cell attachment and proliferation[3,5,12,13,16-18] Until now, several natural and synthetic polymers have been investigated to be used as scaffold materials, but only a very small number is being clinically used in a limited range of tissue engineering applications.

The first stage of tissue engineering, and one of the most important ones, is the design and processing of a porous 3-D scaffold with an interconnected structure of well-distributed pores with appropriate sizes for cell seeding.[19] The methods of manufacturing such scaffolds in a reproducible manner are crucial to their success, and should allow for the necessary scale-up of the developed tissue engineering technology.[4,8] The technique used to manufacture scaffolds for tissue engineering must allow the preparation of scaffolds with complex three-dimensional geometries and adequate porous structure, without affecting the biocompatibility of the material.[3,20]

In this work, several methods based on conventional processing techniques and combined techniques, such as solvent casting and compression with salt leaching, were developed in order to obtain porous structures from starch based polymers, that are suitable for bone tissue engineering applications. The scaffolds obtained by the different methods exhibit different structure/properties combinations that may constitute a promising alternative to currently used biodegradable scaffolds.

2. MATERIALS & METHODS

In this study, two different polymeric blends of corn starch with: i) ethylene vinyl alcohol blends (SEVA-C) and ii) cellulose acetate (SCA), both obtained from Novamont, Italy, were used.

Several blowing agents (BA) were selected for the present study. The first blowing agent selected was Hostatron System P9947, from Hoechst, Germany, which will be designated as blowing agent 1 (BA1). This blowing agent of commercial origin is mainly composed of carboxylic acid that reacts by heating, releasing CO_2 and water at about 200ºC. Two other blowing agents were selected, namely Hydrocerol BIH 70 and Hydrocerol BIH 40, which will be designated by blowing agent 2 (BA2) and blowing agent 3 (BA3), respectively; both were obtained from Clariant, Germany. These blowing agents are based on citric acid and they also release CO_2 and water upon decomposition, which happens around 170ºC. The cytotoxicity of these materials has been tested[21] and the results show a non-cytotoxic behaviour.

2.1. Extrusion with blowing agents

In the extrusion process, the polymers were previously mixed with the blowing agents in a bi-axial rotating drum prior to processing in a twin-screw extruder Carvex, with a die of 12 mm of diameter. This process was optimised for mixtures of the polymer with 10% and 15% (w/w) of blowing agent 1. The weight fraction of blowing agent 2 and 3 necessary to produce the same percentage of porosity was much smaller than the one used with BA1 (between 1 to 2,5%).

2.2. Compression moulding - particle leaching

The compression moulding and particle leaching method was based on blending together a starch based polymer (in the powder form) and leachable particles (in this case, salt particles) of different sizes, from 50μm to 1000μm, in sufficient amounts to provide a continuous phase of a polymer and a dispersed phase of leachable particles in the blend. The blend was then compression moulded into a desired shape. The mould used was specially designed for this purpose, and allowed to obtain discs of 6 cm of diameter and approximately 1 cm of height. The resultant samples were then immersed in distilled water to remove the leachable particles. In all the methods combined with salt leaching

described in this manuscript, the leaching procedure was optimised for “excess leaching” of the salt.

2.3. Solvent-casting/particle-leaching

For the development of the solvent casting and particle leaching method it was necessary to find a solvent capable of dissolving properly the starch based polymers, once that it was found that due to its complex structure (and high molecular weight of the starch) these materials were not soluble, without degradation, in the traditional organic solvents. After performing an all range of solubility tests using several solvents, it was found that at least four solvents were able to dissolve these polymers, namely, acetic acid, dimethylformamide and dimethylsulfoxide. The results shown in this manuscript are referred to SCA (starch with cellulose acetate) dissolved in acetic acid, from Merck, Germany. With this method, the polymers were first dissolved in an appropriate organic solvent and mixed with salt particles of different sizes. In general, it was added a salt weight fraction of 60 to 70% (based on the total mass of polymer and salt). The size of the particles used ranged from 50 to 1000μm. The mixture of the polymeric solution with the salt particles was then poured into a mould (a glass petri-dish of 3cm diameter) and placed in an oven at 37ºC in order to allow a progressive evaporation of the solvent. Finally, when the samples were completely solidified, they were immersed in distilled water, for leaching of the salt particles, and afterwards dried.

2.4. In-situ polymerisation

This innovative so-called in-situ polymerization process was based on a polymerization process developed in our group in order to obtain materials to be used as bone cements or hydrogels[22].

These materials were prepared by adding the liquid phase, constituted by the acrylic monomers ((AA), from Merck), and 1% (w/w) of N-dimethylaminobenzyl alcohol (DMOH), which was used as the activator of the initiation process, to the solid phase, which consisted of SEVA-C powder and 2%(wt/wt) of benzoic peroxide ((BPO), from Merck), which was used as the radical initiator, after purification by fractional recrystallization from ethanol, mp 104 ºC BPO. The leachable NaCl particles were added to the liquid or to the solid phase in order o provide the porosity of the structure. The solid and the liquid phases are then mixed together with a 10% of water with respect to the total weight and poured in a dough state in poly (tetrafluoroethylene) (PTFE) moulds until complete polymerisation take place.

After curing time, about 5 minutes, moulds are placed into the oven at 60ºC overnight to ensure a complete polymerization and then vacuum dried until constant weight was attained. Finally the samples were immersed in water to leach out the salt particles, in order to better simulate the in-vivo application of these materials.

2.5. Materials Characterization

The porous structure of the materials developed, namely the morphology of the pores, their size and distribution and also the interconnectivity between these pores, was characterised by scanning electron microscopy (SEM), in a Leica Cambridge S360. All the samples were previously gold coated in a Sputter Jeol JFC 1100 equipment. The porosity measurements were obtained from the photographs acquired by SEM that were processed using an image analysis software.

The mechanical properties of the developed materials were assessed on compressive experiments in an Instron 4505 universal mechanical testing machine, using a load cell of 50 kN. The compression tests were carried out at a crosshead speed of 2 mm/min (4.7 x 10^{-5} m/s), until obtaining a maximum reduction in samples height of 60%. A minimum of six samples of each type was tested.

The degradation behaviour was assessed after several pre-fixed ageing periods (0,3, 7, 14, 30, 60 and 90 days), in an isotonic saline solution (NaCl 0.154 M). At the end of each degradation period, the samples were removed from the solution, rinsed with distilled water and weighted, to determine the water uptake; one batch of samples was then dried up to exhaustion (6 days at 60ºC) in order to determine the dry weight loss.

3. RESULTS AND DISCUSSION

3.1. Morphology of the porous structures

3.1.1. Extrusion with blowing agents

The porous structure of the samples obtained by extrusion of the polymers combined with blowing agents results from the gases released by decomposition of the BA during processing. Therefore, it is difficult to control the pore size and the interconnectivity between the pores. However, a thin layer of solid material (less than 1 mm in 12 mm thick samples) surrounds the porous structure of the material.

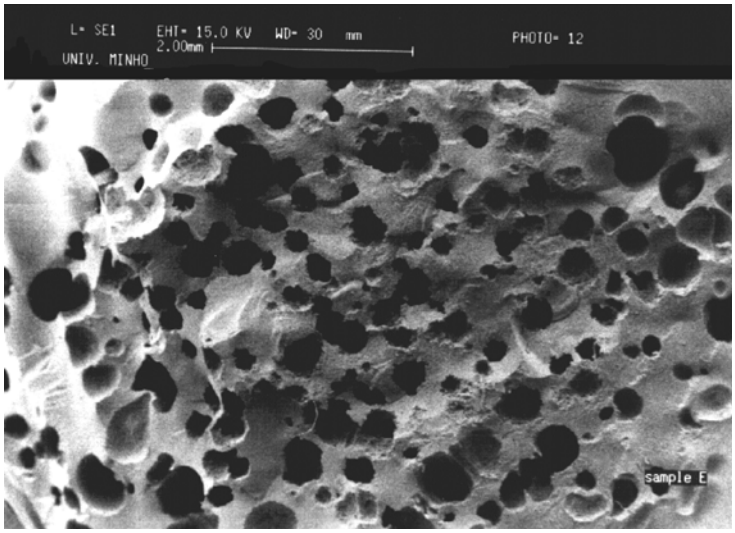

Figure III.1. Scaffold obtained by of SCA extrusion of SCA with 10% of blowing agent 1 (Hostatron 9947)

The extrusion process using blowing agent 1 (figure III.1), produces samples with pore sizes roughly between 50 to 300 μm, but the interconnectivity of the porous structure obtained is still very poor, even with the use of the surfactant agents.

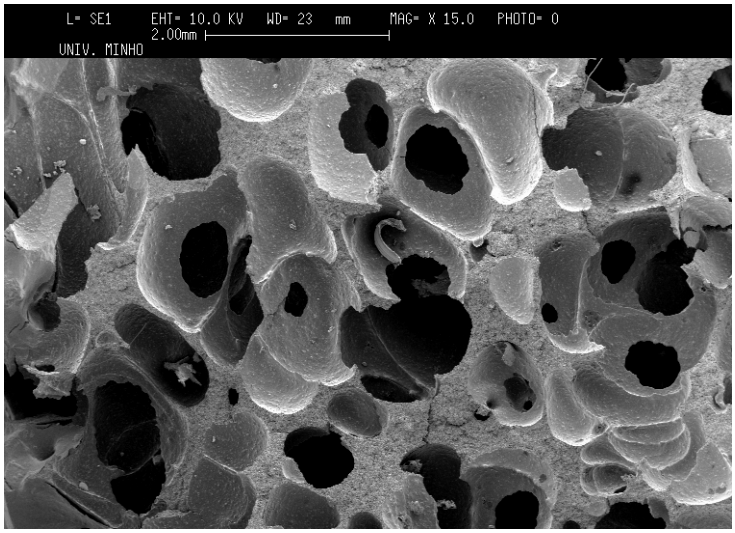

Figure III.2. SCA scaffold obtained by extrusion of SCA with 2.5 % of blowing agent 2 (Hydrocerol BIH 70)

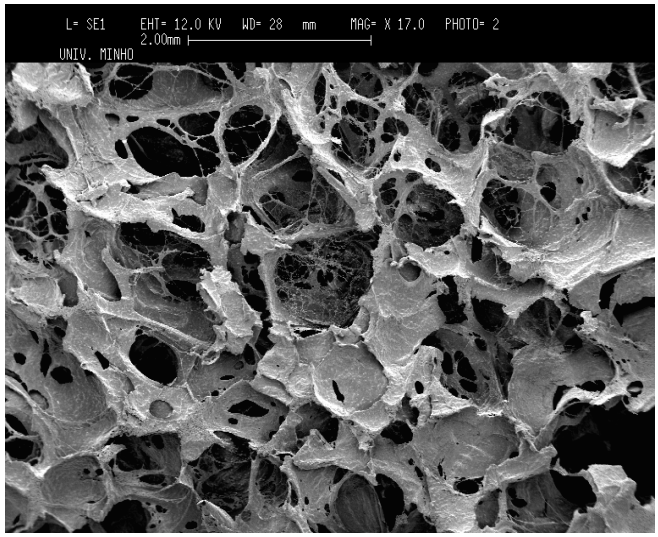

Figure III.3. Scaffold obtained by extrusion of SCA with 2% of blowing agent 3 (Hydrocerol BIH40)

In figures III.2 and III.3 it can be see that by means of using blowing agent 2 and 3 (in both cases hydrocerols), it was possible to obtain higher porosity and significant improvements with respect to the interconnectivity of the porous structures. This becomes much more evident in figure III.3 that corresponds to the materials obtained using the blowing agent 3. Furthermore, such increase in the porosity was obtained using much lower amounts of blowing agent (about 2 wt%). In this case, the pore sizes are in the range of 100 to 500 μm, a range that includes the values that are believed to give the best results in terms of bone cell culture and bone tissue ingrowth by several researchers[17,23,24]. The density of the samples obtained by this process is approximately 0.7-0.8 g/cm^3 (depending mainly on the blowing agent used), leading to a porosity of about 40-50%.

3.1.2. Compression moulding and particle leaching

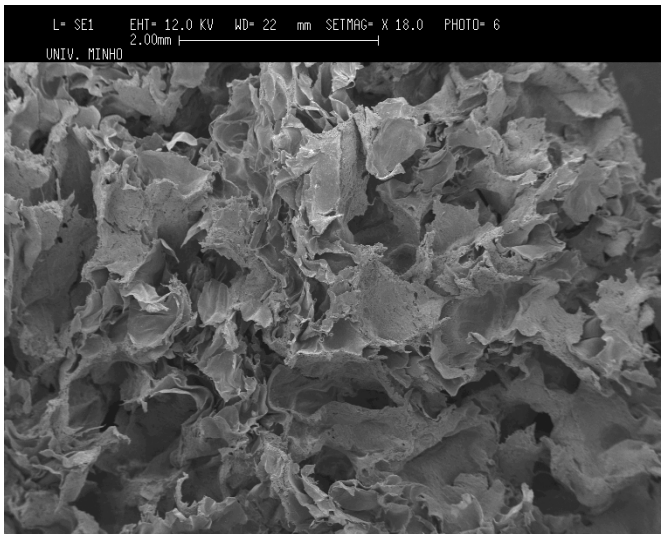

Figure III.4. Scaffold obtained by compression moulding-particle leaching of SCA with 65% of salt.

The compression moulding – particle leaching technique gives rise to structures consisting in an open network of pores throughout the sample (see figure III.4), with sizes ranging from 10 to 500μm. The pores showed a large degree of interconnection, which is very important for cell seeding and growth. These scaffolds present a density of about 0.7g/cm^3, leading to a porosity of about 50%. With this technique it is possible to control the percentage of porosity and the pore size by simply varying the amount and size of the leachable particles.

3.1.3. Solvent casting and particle leaching

The solvent-casting and particle leaching method allowed to obtain an open-porous structure with a good interconnectivity between the pores throughout the entire structure (figure 6). In fact, these scaffolds presented the best interconnectivity achieved so far, with respect to the other processing methods. Furthermore, this method allows for the accurate control of the pore size, distribution of pore sizes and porosity (volume of voids). These parameters of the porous structure can be tailored by varying the size, shape and distribution of the particles and the chosen volume ratio of polymer/particles. The control of scaffold porosity is critical for controlling cellular colonization and organization within an engineered tissue.

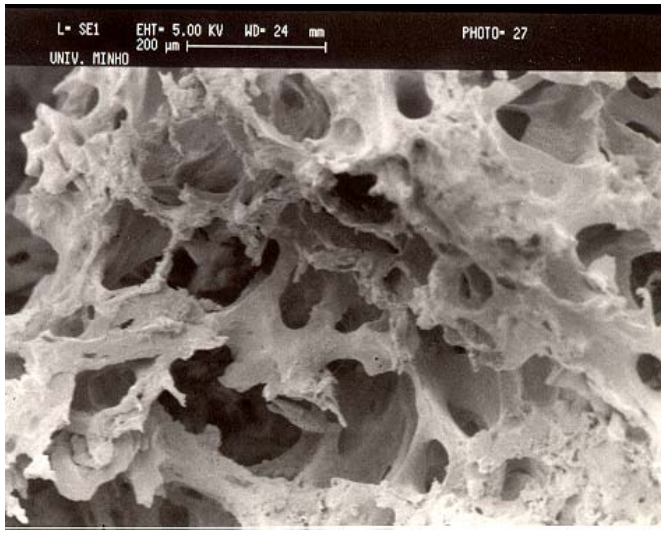

Figure III.5. Scaffold obtained by the solvent casting-particle leaching method

The sample exhibited in figure III.5, present a pore size between 50-300μm. In this cases the samples present a density of about 0.6g/cm^3, which leads to a porosity around 60%.

3.1.4. In situ polymerisation

The innovation introduced by the in-situ polymerisation method of obtaining scaffolds for tissue engineering lies in the fact that it is possible to produce the scaffold in-situ, i.e., it might be possible to inject the scaffold directly into the defect to treat, which can, therefore, take immediately the shape of the defect.

These moldable polymer scaffolds fitting to the three dimensional geometry of specific tissue defects are highly in demand for clinical applications due to the invasiveness of the surgical implantation procedure[25-27] SEM analysis of this scaffolds showed pores ranging in size roughly from 10 to 100μm in diameter (figure 6), but once again, the pore size depends on the salt particles used.

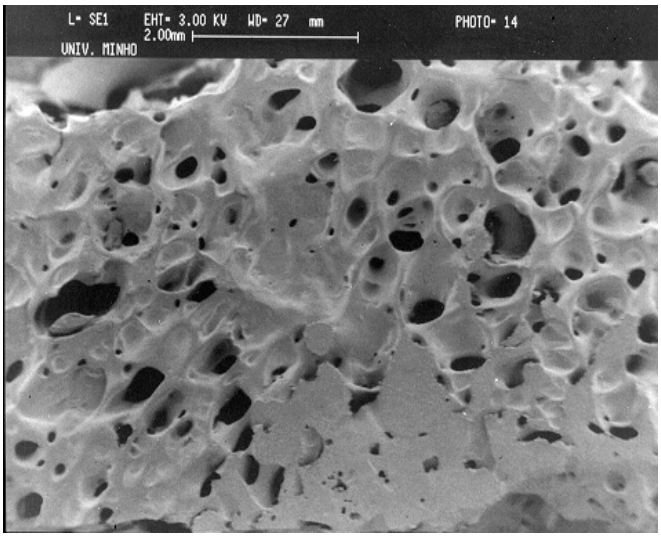

Figure III.6. Scaffold based on SEVA-C obtained by a method based on *in situ* polymerization

3.2. Degradation behaviour

Figures III.7 and III.8 show the water uptake and the weight loss as a function of the degradation period for the scaffolds obtained by the different processing methodologies developed.

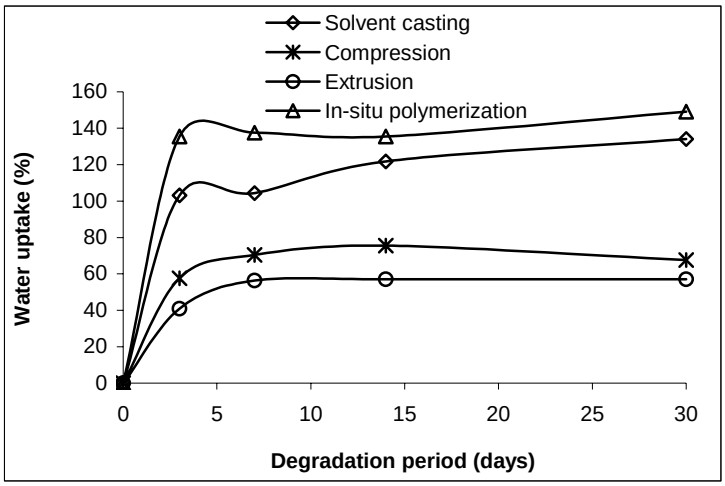

Figure III.7. Water uptake vs. degradation period for SCA based scaffolds obtained by the different processing technologies developed.

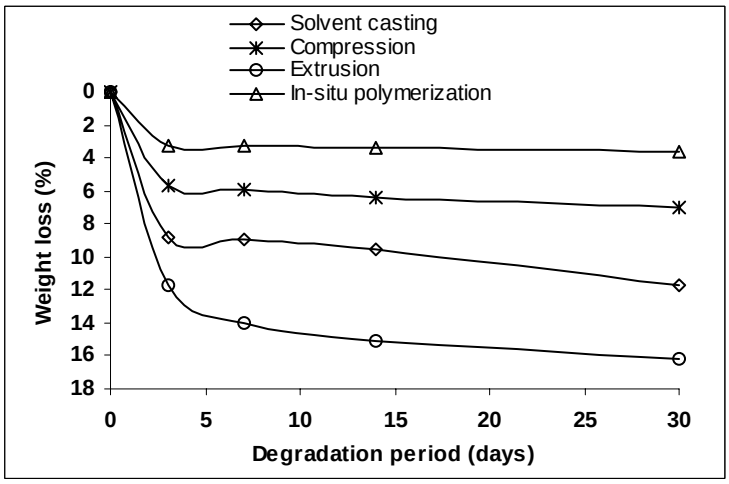

Figure III.8. Weight loss vs. degradation period for SCA based scaffolds obtained by the different processing technologies developed.

As expected, the scaffolds obtained from the different methods presented similar water uptake and degradation profiles although they exhibited different degradation rates and different water uptake ability, according to the processing method and conditions. As expected, the samples obtained using higher blowing agents fraction/ higher salt particles fractions, exhibit higher porosity. The different scaffolds porosity attained by the different method and also to the thickness of the outer solid layer, will have direct influence in the easy of retention and access of the degradation solution in the bulk of the material, respectively, which will therefore influence their degradation behaviour.

The scaffolds obtained by the combination of compression moulding with particle leaching exhibit significantly higher water uptakes, compared to samples obtained by extrusion with blowing agents, which demonstrates the higher porosity and interconnectivity achieved with this method. However, the weight loss of these scaffolds is lower than for the scaffolds obtained by extrusion, which is most probably due to thermo-mechanical degradation undergone by the materials processed by extrusion, which leads to an easier breakdown of the material (associated to leaching of low molecular weight chains).

The scaffolds obtained by the solvent casting and particle leaching exhibit very high water uptakes (more than 100% after only days of immersion in the degradation medium), as compared to samples obtained by all the other processes. This is probably due to the high porosity and interconnectivity achieved with this method.

The scaffolds obtained by the in-situ polymerisation method present the highest water uptake ability, since they have water uptake properties of hydrogels. Their degradation rates are obviously lower than those presented for the above scaffolds, since this final material is composed of a blend of the starch based polymer with acrylic acid. The materials produced by this method are, in fact, not totally degradable, but they might be very useful in situations where it is necessary high mechanical properties and/or in situations where the defect or trauma that is necessary to treat is of difficult access, avoiding highly invasive surgery techniques.

Furthermore, it is also possible to easily incorporate HA in the materials formulation, improving, at the same time, their mechanical properties and biological behaviour. This will be reported in a future paper.

3.3. Mechanical properties

The scaffolds obtained by extrusion with blowing agent 1, present the best compression properties, with a modulus varying from 205 to 230 MPa.

The blowing agents 2 and 3 gave rise to lower mechanical properties, probably due to the higher porosity and interconnection between pores that was achieved with these blowing agents, although these porous structures are more adequate for the final application of the scaffolds. Nevertheless, these scaffolds present very promising mechanical properties when compared to other scaffolds, obtained from other biodegradable polymers, and proposed for use in tissue engineering of bone. For example, PLLA/ hydroxyapatite composite foams, prepared by a process based on phase separation, presented a compression modulus bellow 12 MPa.[5] The results presented on table III.1 might be further improved by reinforcing the scaffolds with hydroxyapatite.

Table III.1. Mechanical properties of the scaffolds obtained by the different processing methodologies developed.

Processing method	Type and percentage of blowing agent or porogen used	Porosity of the scaffolds (%)	Compressive modulus (MPa)	Compressive strenght (MPa)
Extrusion with blowing agents	10% Hostatron 9947 (BA1)	40-50	205.0 ± 9.5	12.0 ± 1.4
	15% Hostatron 9947 (BA1)		217.8 ± 25.8	17.6 ± 0.9
	10% Hostatron 9947 (BA1) + 1% glycerol		230.8 ± 71.0	12.2 ± 2.5
	2% Hydrocerol BIH 70 (BA2)		172.4 ± 35.5	8.7 ± 0.5
	2 % Hydrocerol BIH 40 (BA3)		124.6 ± 27.2	8.0 ± 0.9
Compression molding and particle leaching.	50 % of NaCl particles	50-60	341.6 ± 34.3	67.69 ± 6.2
	65 % of NaCl particles		133.7 ± 20.6	20.56 ± 6.2
Solvent casting and particle leaching.	60-70% of NaCl particles	60-70	170.5 ± 16.09	21.73 ± 1.1

The compressive properties of the scaffolds obtained by compression moulding and particle leaching are also very dependent on the porosity obtained, being in some cases, superior to those obtained by the extrusion process.

As should be expected, the mechanical properties of the scaffolds obtained by the solvent casting and particle leaching method are lower when compared to the properties of the samples obtained by melt-based technologies. However, these properties may be considered very good when compared to scaffolds obtained from other materials by identical processing methods and proposed for the same type of applications. For example, a PLGA scaffold obtained by the solvent casting and particle leaching method, exhibits a modulus of 1.09 MPa [28]

4. CONCLUSIONS

It was possible to develop a wide range of processing methods to obtain starch based tissue engineering scaffolds. These scaffolds present adequate porous structure, that can be tailored according to the intended application, with pore sizes in the range of those which are believed to be the most appropriate for bone cell culturing and/or bone tissue ingrowth. The interconnectivity obtained is dependent on the processing method. In general, the methods based on compression or solvent casting associated with particle leaching, allowed to obtain the best interconnectivity between pores.
With respect to the degradation behaviour, although the profile is similar to all types of samples, the degradation rates can be significantly different, depending on the processing method and on the final porosity obtained.
The mechanical properties of the scaffolds obtained by melt based methods are the highest ones, as compared to the scaffolds obtained by the other methods, but in general all the starch based scaffolds present better mechanical properties than those reported in the literature for scaffolds obtained from other biodegradable polymers and proposed for bone tissue engineering applications.

The methods detailed herein allow for the optimisation of the porous structure, degradation kinetics and mechanical behaviour of starch based scaffolds for a given application within the bone tissue engineering field. Therefore scaffolds obtained from these materials using one of the described methodologies may constitute an important alternative to the materials currently used in tissue engineering.

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Chapter IV

DESIGN AND PROCESSING OF STARCH BASED SCAFFOLDS FOR HARD TISSUE ENGINEERING

Chapter IV

Design and Processing of Starch Based Scaffolds for Hard Tissue Engineering*

Abstract

The design and processing of appropriate porous 3-D scaffolds is one of the most important steps towards the regeneration of damaged tissues/organs using a tissue engineering approach, since most of the cell types require an adequate support in order to form the intended new tissue.

This work reports the development of several processing techniques have been specifically designed for producing biodegradable scaffolds from a range of starch based polymers. The developed methods include melt based processing technologies (based on injection moulding and extrusion using blowing agents), combined techniques based on solvent casting and on compression moulding associated to particle leaching. It has been possible to produce scaffolds that combine an appropriate degradation rate, with controlled porosity and adequate pore sizes, as well as tissue matching mechanical properties. Furthermore, the developed methods have no negative effect on the biocompatible behaviour of the starch based polymers.

*** This chapter is based on the following publication:**

M.E. Gomes, J.S. Godinho, R.L. Reis, A.M. Cunha, "Design and Processing of Starch Based Scaffolds for Hard Tissue Engineering", *Journal of Applied Medical Polymers*, 2002, 6: 75-80

1. INTRODUCTION

Tissue loss or end-stage organ failure resulting from an injury or disease is a major health problem, since the transplantation of tissues or organs in these patients is severely limited by donor scarcity and by the risk of rejection and disease transfer.[1-4]

Tissue engineering has emerged as a promising alternative approach to circumvent the limitations of the existing therapies for the treatment of malfunctioning or lost organs.[1-6]

In this approach, a porous material acts as a temporary scaffold, serving as an adhesive substrate for the implanted cells and simultaneously supporting the formation of the new tissues/organs. Transplanted cells adhere to the scaffold, proliferate, secrete their own extracellular matrices (ECM), and stimulate new tissue formation. During this process, the scaffold gradually degrades and is eventually eliminated.[3,4]

In tissue engineering of bone, the scaffold matrix must serve an additional function:[7-9] it must provide sufficient temporary mechanical support to withstand in vivo stresses and loading. In this case the material must be designed with a degradation rate that ensures that the strength of the scaffold is retained until the tissue engineered transplant is fully remodelled by the host tissue and can assume its structural role.[7-9]

As a consequence, the search for improved biodegradable polymers and for processing techniques to produce scaffolds for hard tissue regeneration, so that physical and chemical properties can be simultaneously optimised, is still an important and very demanding issue in hard tissue engineering research.[10,11]

2. MATERIALS & METHODS

Usually, polymer scaffold processing is divided in two general groups of techniques: melt processing and solvent processing. Melt processing involves heating the polymer above the glass transition temperature (Tg) or the melting temperature (Tm) and depends on melt viscosity. Solvent processing depends on the polymer solubility in various organic solvents and on the solvent volatility.

This section describes several processing methods for producing the scaffolds based on starch based blends with distinct synthetic polymers, namely with cellulose acetate (SCA), ethylene viny alchool (SEVA-C) and polylactic acid (SPLA), all provided by Novamont, Italy. Several blowing agents (BA) were selected for the study, however in this manuscript we only report results obtained with the blowing agents that produced the scaffolds with higher porosities and/or best interconnectivity, namely: Hydrocerol BIH 40 (BA1), from Clariant, Germany and Celogen 780 (BA2) from Uniroyal Chemical.

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2.1. Extrusion and Injection Moulding with Blowing Agents

The polymeric materials were mixed in a rotating drum with one of the blowing agents described above, in amounts from 1% to 15% prior to processing by injection moulding or extrusion. In the injection moulding process it was used a Krauss Maffei KM60-120A) injection moulding machine with a mould which was particularly designed for this application in order to allow maximal expansion and therefore enhance the formation of pores within the polymer melt. In the extrusion process, it was used a Carvex twin-screw extruder with a die diameter of 12 mm.

2.2. Compression moulding - particle leaching

The starch based polymers and the leachable salt particles of different sizes, were blended and then compression moulded into a desired shape. The resultant sample was then immersed in water to dissolve the salt particles, creating a porous structure. The leaching procedure was optimised for “excess leaching” of the salt particles, which corresponded to the immersion of each sample in distilled water during 5 days, changing the water daily.

2.3. Solvent-Casting/Particle-Leaching

The polymers were dissolved in an appropriate organic solvent and then mixed with salt particles of different sizes. When the mixture had solidified, by evaporation of the solvent, the samples were immersed in water to leach out the salt particles.

2.4. Materials Characterization

The porous structure of the materials developed was characterised by scanning electron microscopy (SEM), in a Leica Cambridge S360. All the samples were previously gold coated in a Jeol JFC 1100 sputter coater. The SEM analysis allowed to evaluate the morphology of the pores, their size and distribution and also the interconnectivity between these pores.

The materials were mechanically tested on compression experiments in an Instron 4505 universal mechanical testing machine, using a load cell of 50 kN. Compression testing was carried out at a crosshead speed of 2 mm/min (4.7×10^{-5} m/s), until obtaining a maximum reduction in samples height of 60%. A minimum of six samples of each type was tested.

The degradation behaviour was assessed after several pre-fixed ageing periods (0,3, 7, 14 and 30 days), in an isotonic saline solution (NaCl 0.154 M), at 37ºC. At the end of each degradation period, the samples were removed from the solution, rinsed with distilled water and weighted, to determine the water uptake. Finally 5 of these samples were dried to constant weight (6 days at 60ºC) in order to determine the final dry weight loss and

other 5 samples were dried at room temperature and then mechanical tested (as described above) in order to evaluate the changes in mechanical properties after degradation.

3. RESULTS & DISCUSSION

3.1. Extrusion and Injection Moulding with Blowing Agents:

The porosity of the samples obtained by these processes results from the gases (mainly CO_2) released by decomposition of the blowing agents during processing. Therefore, it is difficult to accurately control the porosity and the pore size obtained. However, the consecutive optimisation of the processing conditions as well as progressive better selection of the blowing agent and mould design, allowed to obtain significant improvements in the percentage of porosity and interconnectivity of the porous structures. This is shown for example, in figure IV.1 that displays a representative SEM microphotograph of the structure of a scaffold obtained by injection moulding of SCA with only 1.5% of the BA2 (Celogen 780). The estimated porosity in this case is about 40-50%, depending mainly on the type and amount of blowing agent used. In addition, the injection mouding and the extrusion processes produce a uniform microporosity throughout the pore walls of the scaffolds (see figure IV.2) which can perform an important role in the transport of nutrients during cell culturing, enabling the proliferation of cells also within the scaffolds and not only on the surface.

The mechanical properties of these scaffolds are mainly affected by the synthetic component of the starch based blend used and obviously on the percentage of porosity of the scaffold (see table IV.1).

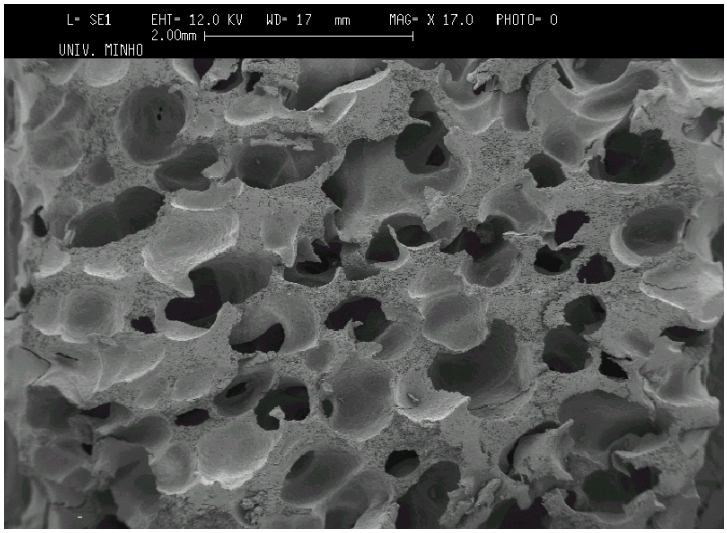

Figure IV.1. Scaffold obtained by injection moulding of SCA with 1.5% of blowing agent 2 (Celogen 780)

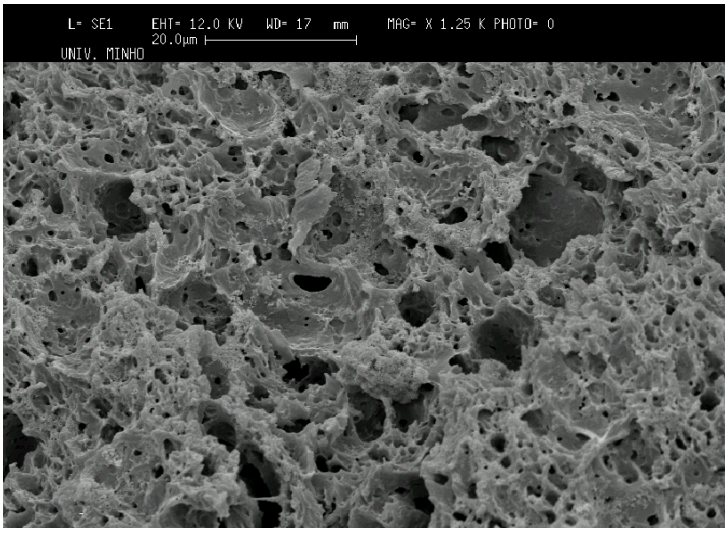

Figure IV.2. Scaffold obtained by injection moulding of SCA with 1.5% of blowing agent 2 (Celogen 780) showing the uniform microporosity of the structure.

3.2. Compression Moulding –Particle Leaching:

The compression moulding and particle leaching technique gives rise to structures on which the porosity and pore size are mainly dictated by the amount and sizes of the leachable particles used and usually with good interconnectivity between pores. Figure IV.3 shows the structure of a scaffold based on SCA obtained by compression moulding with 65% of salt particles. In this case, it was obtained about 65% porosity and the pore sizes were between 10 to 500µ. The compressive strength of these scaffolds are also deeply dependent on the porosity obtained, as shown in table IV.1.

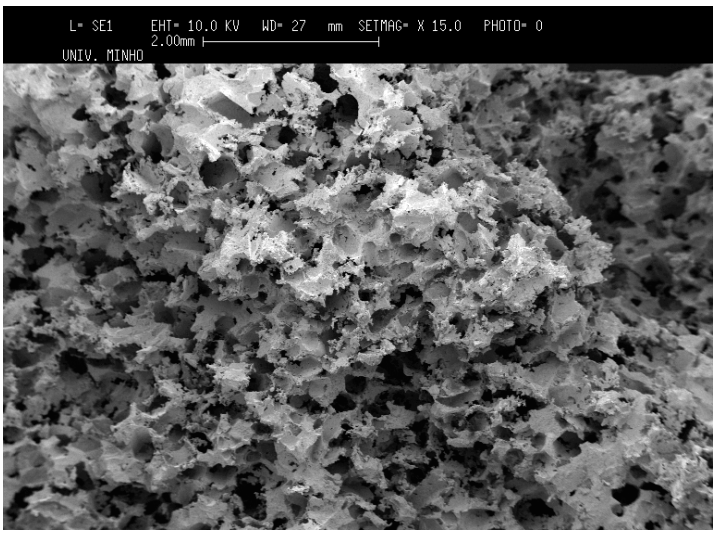

Figure IV.3. Scaffold based on SCA obtained by the solvent casting and particle leaching method using 65% of salt particles.

3.3. Solvent Casting-Particulate Leaching:

The solvent casting and particle leaching method as in the compression moulding with particle leaching, allows for the accurate control of the pore size, distribution of pore sizes and porosity (volume of voids) as these parameters can be tailored by varying the size, shape and distribution of the particles and the chosen volume ratio of polymer/particles. Furthermore, a good interconnectivity between the pores throughout the all structure can be achieved. However, and as expected, the mechanical properties are lower when compared to the properties of the samples obtained by melt-based technologies (see table IV.1).

Table IV.1. Mechanical properties of the SCA (and SEVA-C) based scaffolds obtained by the different methodologies.

Processing Method	Type and percentage of blowing agent or porogen used	Compressive modulus (MPa)	Compressive strength (MPa)
Extrusion	1% Hydrocerol BIH 40 (BA1)	240.1 ± 62.8*	17.28 ± 1.7*
	2% Hydrocerol BIH 40 (BA1)	248.9 ± 39.1*	17.5 ± 1.9*
	3% Hydrocerol BIH 40 (BA1)	249.1 ± 85.1*	18.9 ± 1.1*
	2 % Hydrocerol BIH 40 (BA1)	124.6 ± 27.2	8.0 ± 0.9
Injection moulding	Celogen 780 (BA2)	134.5 ± 39.5	18.4 ± 2.8
Compression molding	50 % of NaCl particles	341.6 ± 34.3	67.69 ± 6.2
	65 % of NaCl particles	133.7 ± 20.6	20.56 ± 6.2
Solvent casting	60-70% of NaCl particles	170.5 ± 16.09	21.73 ± 1.1

* These values refer to SEVA-C based scaffolds; all the other values presented in this table refer to SCA based scaffolds.

4. GENERAL REMARKS

In figures IV.4 and IV.5, the water uptake and degradation behaviour of the scaffolds obtained by the different processing methods described in this study may be compared. The scaffolds obtained from the above described methods presented similar degradation profile. However, they exhibited different degradation rates, according to processing method and processing conditions, especially those that have direct influence in the percentage of porosity, such as the amount of blowing agent and or amount of leachable particles. In general, the methods that give rise to scaffolds with higher porosity and interconnectivity exhibit higher water uptake. However, the degradation rate is not only influenced by the porosity of the structure but also by other aspects related to the processing method. For example, the scaffolds obtained by compression moulding and particle leaching exhibit higher water uptake than those obtained by extrusion and injection moulding with blowing agents, but their weight loss is lower. This is most problably due to the thermo-mechanical degradation undergone by the materials processed by injection

moulding and extrusion, which leads to an easier breakdown of the polymeric chains. In addition to this, the lower porosity and poor interconnectivity of the samples obtained by these processes, but particularly with the injection moulding, may contribute to faster degradation rates due to enhanced sutocatalysis in those scaffolds, which are unable to evacuate acidic degradation by-products.

The mechanical properties of the scaffolds do not exhibit signifficant decreases after *in vitro* degradation during the first 30 days as shown, for example, for SPLA based scaffolds obtained by extrusion and compression moulding (see figure IV.5). The mechanical properties of these scaffolds, namely the compressive modulus, are in the range of those reported for human trabecular bone, and the fact that it is decreased less than 30% in the first 30 days of in-vitro degradation is determinant for their application as bone tissue engineering scaffolds.

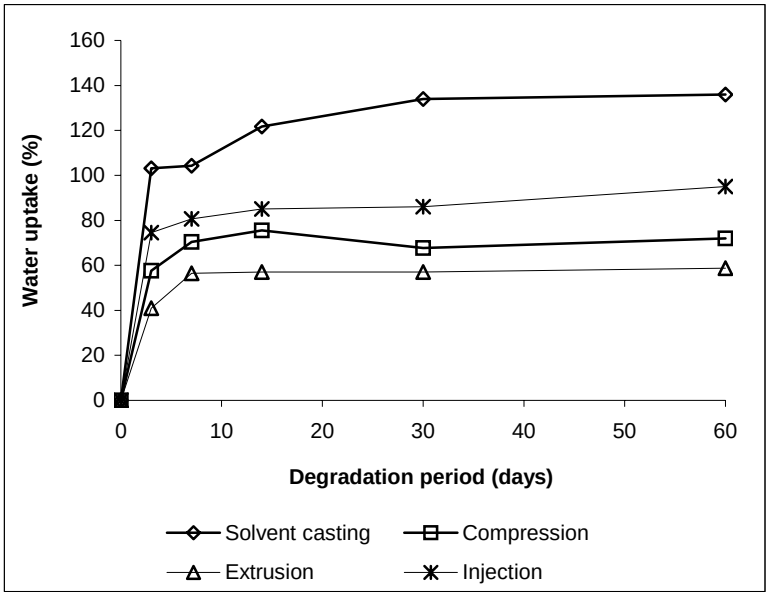

Figure IV.4. Water uptake vs. degradation period for SCA based scaffolds obtained by the different processing technologies developed. Standard deviations are between 3.1 to 5.36% (not shown for easier visualization of the displayed results).

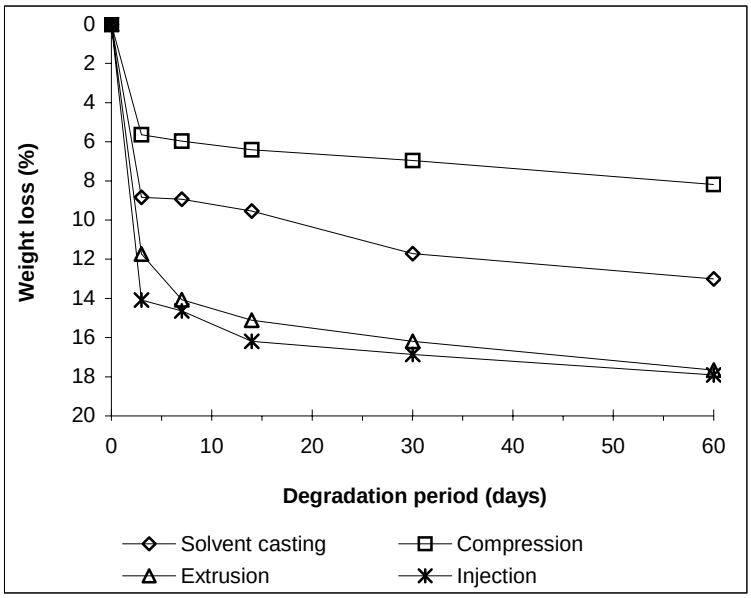

Figure IV.5. Weight loss vs. degradation period for SCA based scaffolds obtained by the different processing technologies developed. Standard deviations are between 0.38 to 1.18% (not shown for easier visualization of the displayed results).

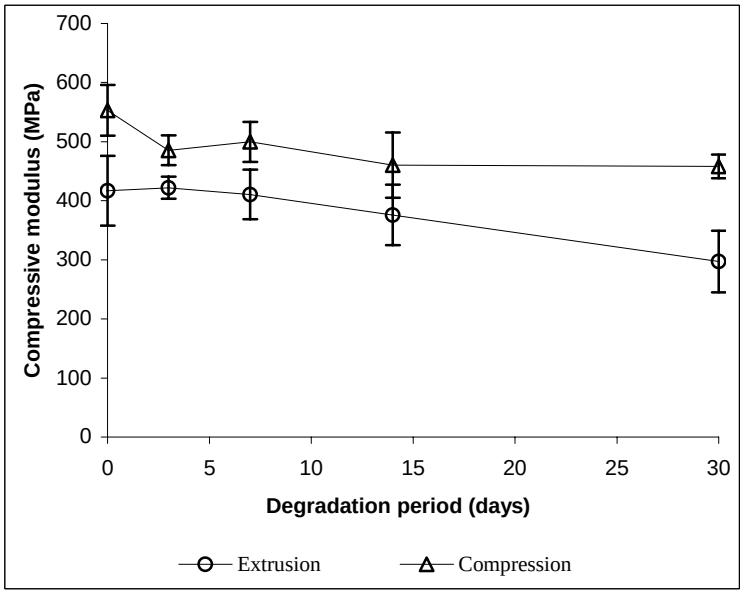

Figure IV.6. Compressive modulus of SPLA based scaffolds obtained by two different processing technologies after several degradation periods in a saline solution.

The biological behaviour of starch based polymers has been the subject of several different *in vitro* and *in vivo* studies [12-14]. All these studies have shown that these polymers

exhibit a biocompatible behaviour, which is not affected under controlled processing conditions. More recently, preliminary cell culture studies have demonstrated that the starch based scaffolds produced by the methodologies described herein allow for the proliferation and growth of human osteoblasts[15]. Although it is necessary to further test these materials with respect to their ability to act as a support for the growth and differentiation of cells, these preliminary results indicates the potential of the developed porous materials for use as bone tissue engineering scaffolds.

5. CONCLUSIONS:

Several processing methods to obtain starch based scaffolds were developed. These methodologies allow to tailor, to an extended degree, the pore sizes and pore structure of the scaffolds as well as their degradation rates since it was shown that the degradation rates can be significantly different, depending on the processing method and on the final porosity obtained.

Furthermore, although only few data on mechanical properties of scaffolds for tissue engineering is found in the literature, it is possible to conclude that mechanical properties of all the tested scaffolds are very promising, when compared to scaffolds obtained from other biodegradable polymers. In addition, these properties are not significantly affected in the first 30 days of *in vitro* degradation, which suggests that the scaffold will be able to provide the necessary structural support in the first period of implantation.

In conclusion, all these results, both from a materials science and a biological perspective, are very promising for the future application of starch based biodegradable polymers as tissue engineering scaffolds.

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Chapter V

EFFECT OF FLOW PERFUSION ON THE OSTEOGENIC DIFFERENTIATION OF BONE MARROW STROMAL CELLS CULTURED ON STARCH BASED THREE-DIMENSIONAL SCAFFOLD

Chapter V

Effect of Flow Perfusion on the Osteogenic Differentiation of Bone Marrow Stromal Cells Cultured on Starch Based Three-Dimensional Scaffold

Abstract

This study aims to investigate the effect of culturing conditions (static and flow perfusion) on the proliferation and osteogenic differentiation of rat bone marrow stromal cells seeded on two novel scaffolds exhibiting distinct porous structures. Specifically, scaffolds based on SEVA-C (a blend of starch with ethylene vinyl alcohol) and SPCL (a blend of starch with polycaprolactone) were examined in static and flow perfusion culture. SEVA-C scaffolds were formed using an extrusion process, while SPCL scaffolds were obtained by a fiber bonding process. For this purpose, these scaffolds were seeded with marrow stromal cells harvested from femoras and tibias of Wistar rats and cultured in a flow perfusion bioreactor and in 6-well plates for 3, 7 and 15 days. The proliferation and alkaline phosphatase activity patterns were similar for both types of scaffolds and for both culture conditions. However, calcium content analysis revealed a significant enhancement of calcium deposition on both scaffold types cultured under flow perfusion. This observation was confirmed by Von Kossa stained sections and tetracycline fluorescence. Histological analysis and confocal images of the cultured scaffolds showed a much better distribution of cells within the SPCL scaffolds than the SEVA-C scaffolds, which had limited pore interconnectivity, under flow perfusion conditions. In the scaffolds cultured under static conditions, only a surface layer of cells was observed. These results suggest that flow perfusion culture enhances the osteogenic differentiation of marrow stromal cells and improves their distribution in three-dimensional starch-based scaffolds. They also indicate that scaffold architecture and especially pore interconnectivity affect the homogeneity of the formed tissue.

*** This chapter is based on the following publication:**

ME Gomes, VI Sikavitsas, E Behravesh, RL Reis, AG Mikos. *Effect of Flow Perfusion on the Osteogenic Differentiation of Bone Marrow Stromal Cells Cultured on Starch Based Three-Dimensional Scaffolds*. Journal of Biomedical Materials Research (2003) **67A**:87-95

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1. INTRODUCTION

One of the most widely studied tissue engineering approaches involves the seeding and extended *in vitro* culturing of cells within a biodegradable scaffold prior to implantation. The bioresorbable scaffold must be biocompatible and porous to facilitate rapid vascularization and growth of newly formed tissue.[1-8] During the *in vitro* culture period, the seeded cells proliferate and secrete tissue specific extracellular matrix (ECM). Following implantation, the scaffold gradually degrades and is eventually eliminated from the body. [1-8]

The selection of an appropriate scaffold material is a primary consideration in such a tissue engineering strategy.[9] Besides the obvious demands of biocompatibility and biodegradability, an ideal tissue engineering scaffold should have appropriate mechanical properties[1,10-14] and a suitable degradation rate.[2,10,12-15] Furthermore, the scaffold must possess adequate porosity, interconnectivity and permeability to allow the ingress of cells and nutrients[12-15] as well as the appropriate surface chemistry for enhanced cell attachment and proliferation.[3,12,13,16] Several biodegradable polymers have been proposed to be used as three-dimensional scaffolds for bone tissue engineering, including a new range of natural origin polymers based on starch.[17] Starch-based polymers are degradable and biocompatible polymers,[18-20] with distinct structural forms and properties that can be tailored by the synthetic component of the starch-based blend, their processing methods, and the incorporation of additives and reinforcement materials. For this reason, together with their low cost and abundance of raw materials, starch-based polymers have been suggested for a wide range of biomedical applications.

Another important consideration for tissue engineering approaches based on *in vitro* cell culture is the cell source and the ability to control cell proliferation and differentiation. Marrow stromal cells constitute a potential autogenous source of cells for bone tissue engineering as they can be expanded, differentiated into osteoblasts, and used to seed the scaffolds.[21]

Besides the selection of the scaffold material and the cell source, several other issues should be considered, including the optimization of the *in vitro* culturing system. Static culturing conditions often result in inhomogeneous cell distribution, confining the majority of the cells to the outer surfaces of the scaffold. Accordingly, an inhomogeneous distribution of the extracellular matrix commonly results.[22-24] In order to overcome this limitation, several bioreactors have been developed,[22-28] attempting to maintain a uniform distribution of cells on the scaffolds, to provide adequate levels of oxygen, nutrients, cytokines and growth factors, and to expose the cultured cells to mechanical stimuli. Most bioreactors used in bone tissue engineering applications achieve good mixing of the culturing media near the construct outer surface, but not to its interior. This inability

represents a major drawback, particularly in the culturing of scaffolds for the reconstruction of large bone defects. Our laboratory has developed a flow perfusion bioreactor which provides uniform flow to the interior and exterior of the cultured scaffolds.[26]

In this study we have investigated the influence of the cell culturing conditions generated by this flow perfusion bioreactor on the proliferation and osteogenic differentiation of rat bone marrow stromal cells seeded into two types of starch-based scaffolds. This study addresses the following questions: i) Are starch based scaffolds able to support adhesion and proliferation of rat bone marrow cells? ii) Does flow perfusion allow for enhanced osteogenic differentiation and homogenous spatial distribution of the seeded cells? iii) Does the different porosity and pore architecture of these scaffolds influence the proliferation, differentiation, and distribution of cells under flow perfusion culture conditions?

2. MATERIALS AND METHODS

2.1. Scaffold preparation and characterization

Two different types of starch based polymer scaffolds were used in this study: i) a scaffold based on SEVA-C (a 50/50%wt blend of starch with ethylene vinyl alcohol) obtained by extrusion with a blowing agent and ii) a scaffold based on SPCL (a blend of starch with polycaprolactone, 30/70%wt) obtained by a fiber bonding process. Further information on starch based polymer scaffolds and their processing can be found elsewhere.[17] The morphology of the scaffolds was characterized by microcomputerized tomography (μCT) (ScanCo Medical μCT 80, Bassersdorf, Switzerland) at a resolution of 10 μm. All samples were cut into discs of approximately 8 mm diameter and 1.5 to 2 mm height and sterilized using ethylene oxide. Prior to cell seeding, the scaffolds were immersed in 30 ml of serum-free media in 50 ml tubes. Air was removed from their pores by generating vacuum with a 30 ml syringe equipped with an 18 gauge needle. The scaffolds were left in serum-free media overnight to allow swelling.

2.2. Isolation and expansion of rat bone marrow cells

Rat bone marrow stromal cells were isolated and cultured using methods previously described.[29] Briefly, cells were obtained from the femoras and tibias of male Wistar rats with weights ranging from 125 to 149 g (Harlan, USA). The epiphyses were cut off and the diaphyses flushed with 5 ml of complete media (α-MEM (Minimal Essential Medium) Eagle, Sigma, USA), supplemented with 10 % FCS (Fetal Calf Serum, Gemini, USA), 50

μg/ml ascorbic acid (Sigma, Chemical Co., St. Louis, MO, USA), 50 μg/ml gentamycin, 100 μg/ml ampicillin, 0.3 μg/ml fungizone, 10 mM β-glycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma)). Cells were cultured in complete media in a humidified atmosphere of 5 % CO_2 at 37°C for 6 days.

2.3. Cell seeding on starch-based scaffolds

After 6 days of primary culture, the cells were detached using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA, Sigma), concentrated by centrifugation at 1500 rpm for 5 min and resuspended in complete media. Subsequently, the scaffolds (n=18 for flow and n=18 for static culture, for each scaffold type) were inserted into cassettes that were placed in 6-well plates. Each scaffold was then seeded with 300 μl of a cells suspension containing $5x10^5$ cells and incubated for 2 hours. Then 10 ml of complete media was added to each well. The seeded scaffolds were further incubated overnight to allow for cell attachment. The following day, seeded scaffolds were placed into fresh 6-well plates for static culture conditions or into the flow perfusion bioreactor and cultured in complete media for 3, 7, and 15 days (6 scaffolds per culture condition and per culture time).

2.4. Cell culturing: The flow perfusion culture system

The flow perfusion bioreactor is described in detail elsewhere.[26] Briefly, the bioreactor consists of 6 flow chambers, each one containing a cassette in which the scaffold is press-fitted. Gas permeable silicon tubing connects each flow chamber with a peristaltic pump and a medium reservoir. Each chamber has its own independent pumping circuit, but all pumps draw media from a common reservoir. For these experiments, culture media was pumped continuously at a flow rate of 0.3 ml/min through the cell/scaffold construct cassette/housing unit and recirculated back to the reservoir. The total volume of medium in the flow system was 210 ml. In the static culture, 10 ml was added to each scaffold. In both culture systems, media was changed every 3 days.

The entire flow perfusion bioreactor was maintained in an environment of 37°C with 5% CO_2. At the end of each culturing period, the cell/scaffold constructs were rinsed with phosphate buffered saline (PBS) and stored at -70°C in 10 ml tubes containing 1.4 ml of milliQ water for DNA, calcium, and alkaline phosphatase (ALP) analysis. At each time point, two scaffolds from each group were retrieved; one was fixed in a solution of 2.5% gluteraldehyde for SEM analysis and the other in a formalin solution for histological evaluation.

2.5. Characterization of the cultured scaffolds

2.5.1.Cellularity of scaffolds

The DNA content of each scaffold was measured using a PicoGreen DNA Quantification Kit (Molecular Probes). The samples were allowed to thaw at room temperature and then they were sonicated for roughly 15 min. A description of the assay can be found elsewhere.[30] The cellularity of each scaffold was then calculated by correlation with the DNA of a known amount of marrow stromal cells.

2.5.2. Alkaline phosphatase activity

Alkaline phosphatase activity was measured using a Sigma Diagnostic Kit #104; a colorimetric endpoint assay which measures the conversion of p-nitrophenol phosphate to p-nitrophenol by the enzyme alkaline phosphatase.[26]

2.5.3. Calcium content of scaffolds

Cell/scaffold constructs were incubated overnight in 1 N acetic acid to dissolve the deposited calcium. The calcium content was then measured using the Sigma Diagnostic Kit #587. This colorimetric endpoint assay measures the amount of calcium-cresolphthalein complexone formed when cresolphthalein complexone binds to free calcium in an alkaline solution. The amount of deposited calcium was expressed as mg of Ca^{2+} equivalents per scaffold.[26]

2.5.4. Scanning electron microscopy (SEM)

For SEM analysis the samples were fixed in a solution of 2.5% glutaraldehyde (in PBS), dehydrated in a gradient series of ethanol solutions, dried with tetramethylsilane, and sputter coated with gold (Jeol JFC 1100, Jeol, USA). Samples were then observed using a scanning electron microscope (Leica Cambridge S360, Leica Cambridge, UK).

2.5.5. Confocal microscopy

To visualize the distribution of cells within the scaffolds, samples cultured for 15 days (previously fixed with glutaraldehyde) were cut in half (to expose the interior cross section), rinsed with PBS, incubated with picogreen dye (0.1%) for at least 15 min, and then observed under a confocal microscope (Zeiss LSM Axiovert, Carl Zeiss, Germany). Depth projections of the surface (up to 300 μm) were obtained and the cells were pseudo-coloured as a function of their distance from the surface.

2.5.6. Histology and imaging of tetracycline fluorescence

The cultured samples were rinsed with PBS, fixed in formalin, rinsed with water and embedded in frozen tissue embedding media (HistoPrep™, Fisher Diagnostic, USA). Sections of approximately 30 μm were obtained using a cryotome (Microm 505) and stained with hematoxylin and eosin for histological evaluation. For visualization of mineralized tissue, additional sections were exposed to a 5% silver nitrate solution under UV light for 25 min and counter stained with a safranin O solution (0.5%). Mineral deposition was also observed in unstained sections under fluorescent light after adding tetracycline-HCl (10 μg/ml) to the culture media as described previously.[23] All the histological sections were observed with a light microscope Nikon E600 equipped with a Sony DXC-950P CCD camera and a fluorescence lamp.

2.5.7. Statistics

Results are presented as means ± standard deviation. Multiple pairwise comparisons were performed using the Tukey-Kramer method with a significance level of 95%.

3. RESULTS AND DISCUSSION

3.1. Characterization of the scaffolds

The SPCL scaffold has a typical fiber-mesh structure, with a fiber diameter roughly 181 μm, with highly interconnected pores and a porosity of approximately 75%, as determined by μCT analysis. (Figure V.1a). The SEVA-C based scaffolds were obtained by extrusion with a blowing agent creating pores within the polymer melt as the blowing agent releases CO_2. The pores are not completely interconnected, as shown on the μCT scan of this scaffold (Figure 1b). The porosity of these scaffold was approximately 60% as determined by μCT analysis.

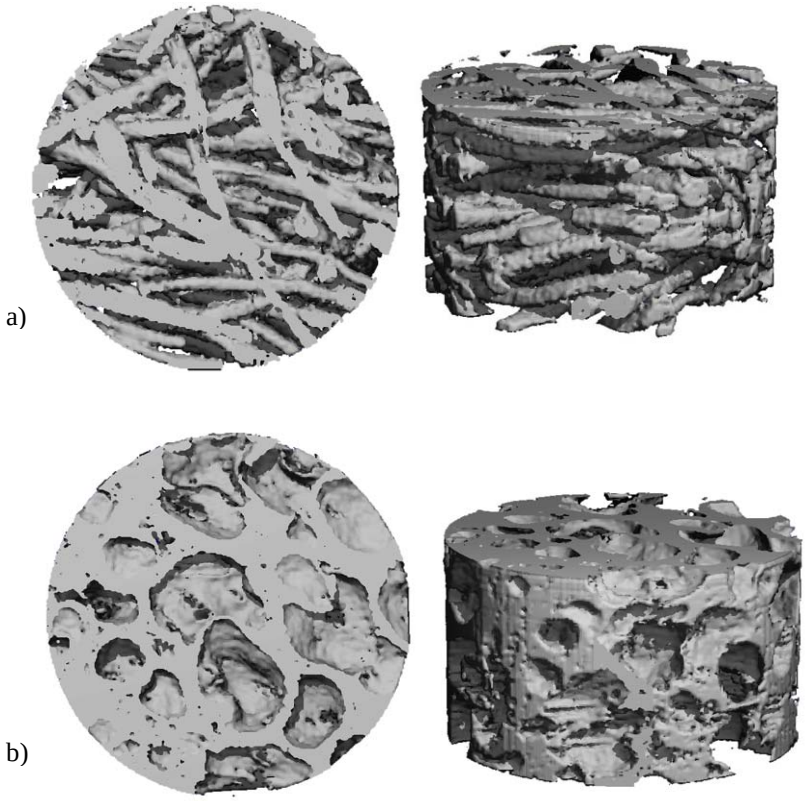


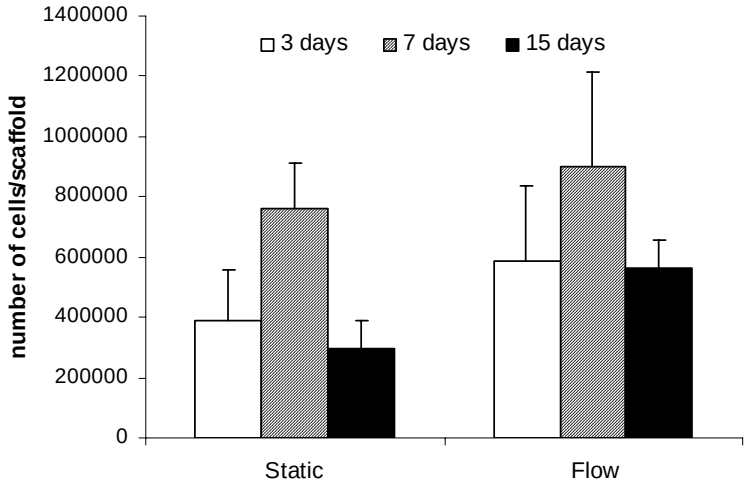
Figure V.1. Representative μCT scans of (a) SPCL based scaffold (top and side view) and (b) SEVA-C based scaffold (top and side view).

3.2. DNA analysis

Figures V.2a and V.2b depict the number of cells present in each scaffold at each time point, calculated from DNA measurements. In both static and flow perfusion cultures, cell proliferation occurred during the first week of culture. These results agree with the initial period of osteoblastic development characterized by active cellular proliferation.[31] Similar cellular growth patterns were observed for both static and flow cultures. Flow perfusion appears to enhance cell proliferation when compared to static culture. This trend was found to be significant ($p<0.05$) for SEVA-C scaffolds cultured for 15 days under flow perfusion. However, when cell proliferation between the two scaffolds cultured in flow perfusion is compared, enhanced proliferation is observed on the SPCL fiber meshes. This may be related to the different synthetic components of the starch-based blend or to the higher porosity and interconnectivity of the SPCL scaffolds.

During the second week of culture, the cellularity of all scaffolds did not increase further. This period corresponds to the stages of matrix maturation, late osteoblastic differentiation, and mineralization. Similar cell proliferation patterns, characterized by an increase in cell number during the first period of culture followed by a period where the cell number

remained constant or even decreased have been observed in earlier studies with marrow stromal cells seeded into titanium fiber mesh scaffolds and cultured under flow perfusion and static conditions.[23,26]



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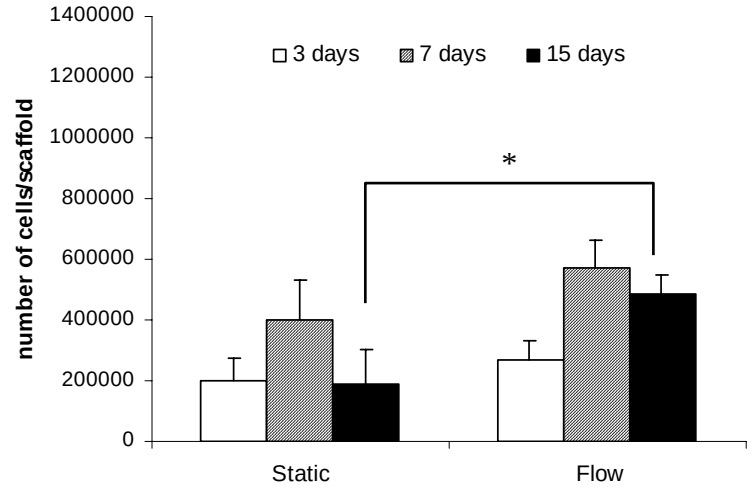


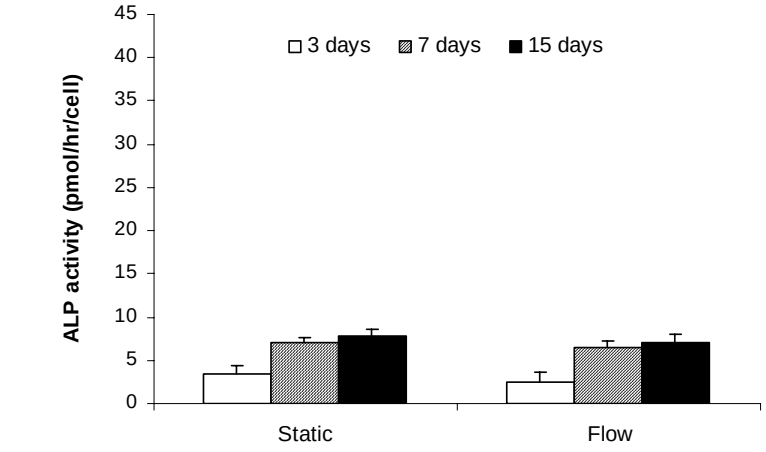
b)

Figure V.2. Number of cells on (a) SPCL fiber meshes and (b) SEVA-C based scaffolds after 3, 7 and 15 days of culture under static and flow perfusion conditions. Error bars represent means ± standard deviation for n = 3-5. Asterisk (*) indicates that the cellularity of SEVA-C scaffolds cultured under flow perfusion conditions after 15 days of culture was significantly higher ($p<0.05$) than the cellularity of scaffolds cultured under static conditions for 15 days.

3.3. Alkaline phosphatase analysis

The alkaline phosphatase (ALP) activity of marrow stromal cells has been shown to indicate the commitment of these cells towards the osteoblastic lineage[31] and usually

reaches a maximum that coincides with the early osteoblastic differentiation stage of marrow stromal cells. After this period, the ALP activity usually decreases and mineralization starts to take place. In this study, a continuous increase in ALP activity was observed during the 15 days of culture on both scaffold types and culture conditions (Figure V.3). For the SPCL fiber meshes, the ALP (normalized to reflect the ALP activity per cell) was not statistically different between flow perfusion and static cultures. However, the ALP activity in SEVA-C scaffolds cultured under flow conditions after 15 days was higher ($p<0.05$) than ALP activity in static culture conditions for the same time period.



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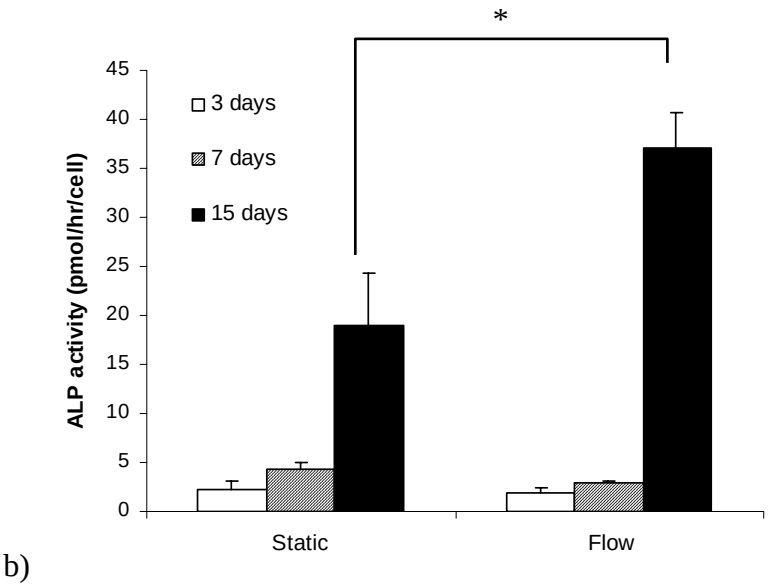
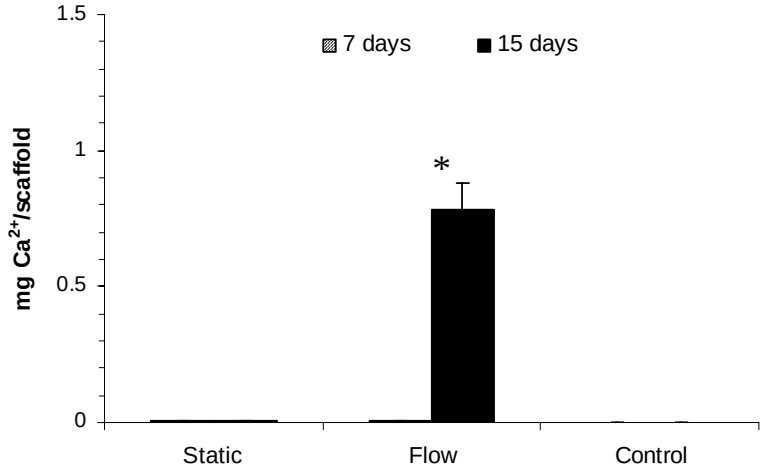


b)

Figure V.3. Normalized ALP activity of marrow stromal cells after 3, 7 and 15 days of culture on (a) SPCL fiber meshes and (b) SEVA-C based scaffolds under static and flow perfusion conditions. Error bars represent means ± standard deviation for n = 3-5. Asterisk (*) indicates that the ALP activity of SEVA-C scaffolds cultured under flow perfusion conditions after 15 days of culture was significantly higher ($p<0.05$) than the ALP activity of scaffolds cultured under static conditions for 15 days.

3.4. Calcium deposition



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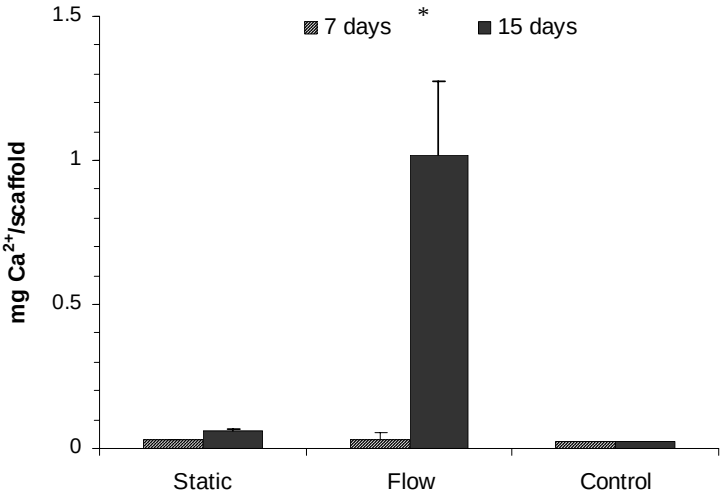

b)

Figure V.4. Calcium deposition on (a) SPCL fiber meshes and (b) SEVA-C based scaffolds after 7 and 15 days of culture under static and flow perfusion conditions. Control samples without cells were also "cultured" under static conditions. Error bars represent means ± standard deviation for n = 3-5. Asterisk (*) indicates that calcium deposited on the scaffolds cultured under flow perfusion conditions after 15 days of culture was significantly higher ($p<0.05$) than calcium deposited on scaffolds cultured under static conditions for 15 days (and scaffolds cultured under flow perfusion conditions for 7 days).

Calcium deposition in cultures of osteoblastic cells is a marker of their full maturation. Calcium measurements (Figure V.4) showed that during the first week of culture practically

no calcium deposition had occurred in both culture conditions. In contrast, after 15 days, a dramatic increase in calcium deposition was observed on both types of scaffolds cultured under flow perfusion, clearly suggesting that flow perfusion is responsible for the enhanced mineralization of marrow stromal cells. Possible mechanisms responsible for the observed enhanced mineralization include the exposure of the seeded cells to fluid shear induced mechanical stimulation and the mitigation of potential nutrient transport limitations experienced by the cells cultured under static conditions.

Enhanced mineralization under flow perfusion appeared in both scaffold types in agreement with earlier studies involving the culture of marrow stromal cells on titanium fiber meshes under flow perfusion with similar shear forces.[26] From the porosity and pore size of the scaffolds, a mean shear stress could be estimated for fluid flow through the pores.[25] Assuming a cylindrical pores model approximation for the geometry of the scaffold porosity, the shear forces experienced by the seeded cells in both scaffolds were estimated to be on the order of 0.1 dyn/cm^2.

3.5. Confocal Microscopy

Confocal images obtained from depth projections of the surfaces of SPCL fiber meshes after 15 days showed the formation of cell monolayers on the surface of the scaffolds cultured under flow (Figure V.5a) and static (Figure V.5c) conditions, but the images suggested the presence of a thicker layer of cells on samples cultured in the flow perfusion bioreactor. The images obtained from the transversal section of the same samples demonstrated that the flow culture conditions (Figure V.5b) allow for a much better distribution of cells inside the fiber meshes than the static culture conditions (FigureV.5d).
In the SEVA-C based scaffolds, the formation of cell monolayers on the surface of the scaffolds cultured under flow (Figure V.6a) and static conditions (Figure V.6c) was apparent. However, the images obtained from the transversal section of the samples (Figures V.6b and V.6d) demonstrated that, in this case, the limited pore connectivity of these scaffold did not allow the cells to spread throughout the scaffold interior. Nevertheless, in the samples cultured in the flow perfusion bioreactor, it was possible to visualize a small number of cells in the interior of the scaffold, indicating the existence of a preferential flow pathway through these scaffolds that allowed the presence of cells in specific locations.

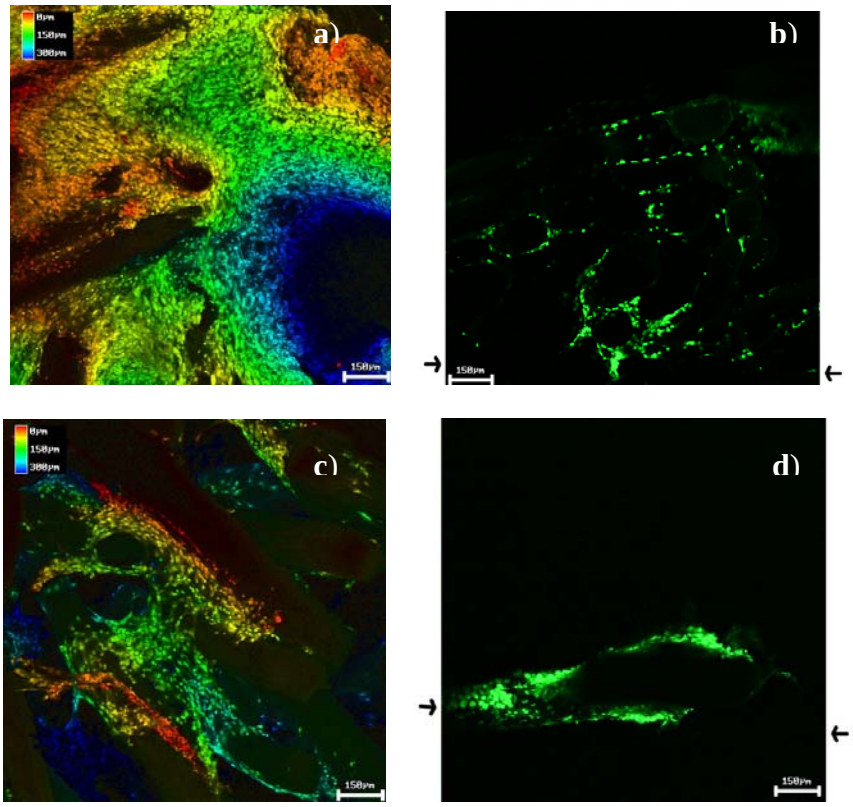

Figure V.5. Confocal microscopy images of SPCL fiber meshes cultured for 15 days obtained from depth projections of the top surface (where cells were seeded) of samples cultured under (a) flow perfusion or (c) static conditions, and showing cell distribution along a transversal section from the surface (indicated by the arrow) to the interior of the scaffolds cultured under (b) flow perfusion or (d) static conditions.

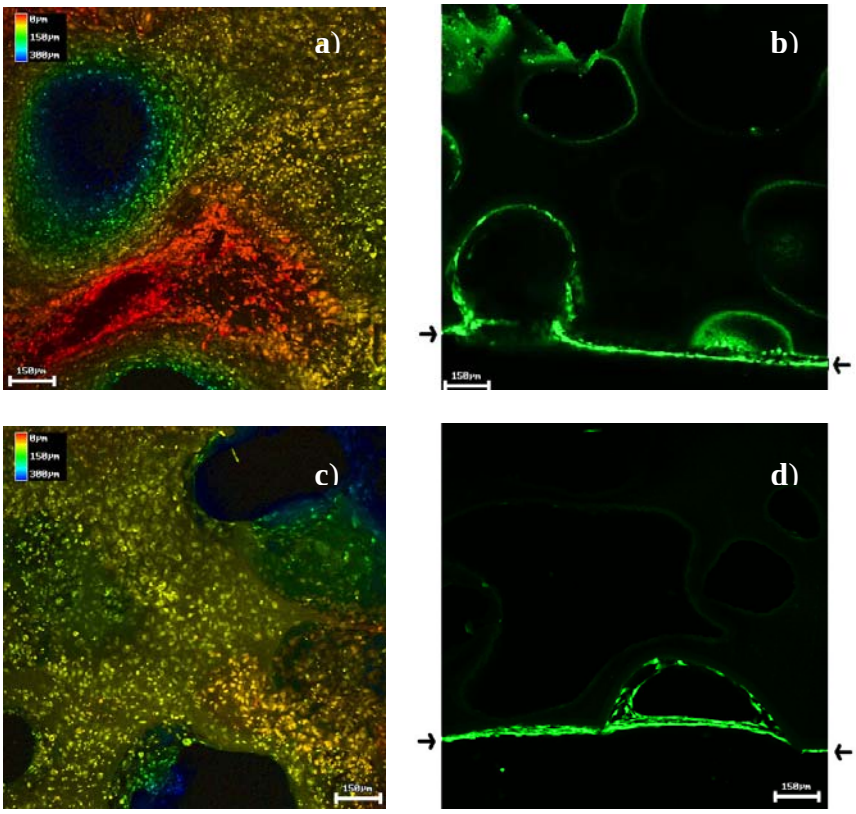

Figure V.6. Confocal microscopy images of SEVA-C based scaffolds cultured for 15 days obtained from depth projections of the top surface (where cells were seeded) of samples cultured under (a) flow perfusion or (c) static conditions, and showing cell distribution along a transversal section from the surface (indicated by the arrow) to the interior of the scaffolds cultured under (b) flow perfusion or (d) static conditions.

3.6. Histological evaluation

3.6.1. Hematoxylin and eosin staining

Histological sections of SPCL scaffolds after 15 days culture (Figures V.7a and V.7b) demonstrated the formation of a cell layer on the surface of the cultured fiber meshes, but not in the interior, under static conditions. In contrast, the constructs cultured under flow conditions (Figures V.7c and V.7d) exhibited a much better cell distribution. The histological analysis of SPCL scaffolds cultured for 15 days was in agreement with the conclusions drawn from confocal imaging. Once again, the presence of cells inside the scaffolds cultured under flow perfusion was observed, while scaffolds cultured under static

conditions resulted only in a surface layer of cells (Figure V.7). These results confirm that flow perfusion culture enhances cell distribution in three-dimensional starch-based scaffolds.

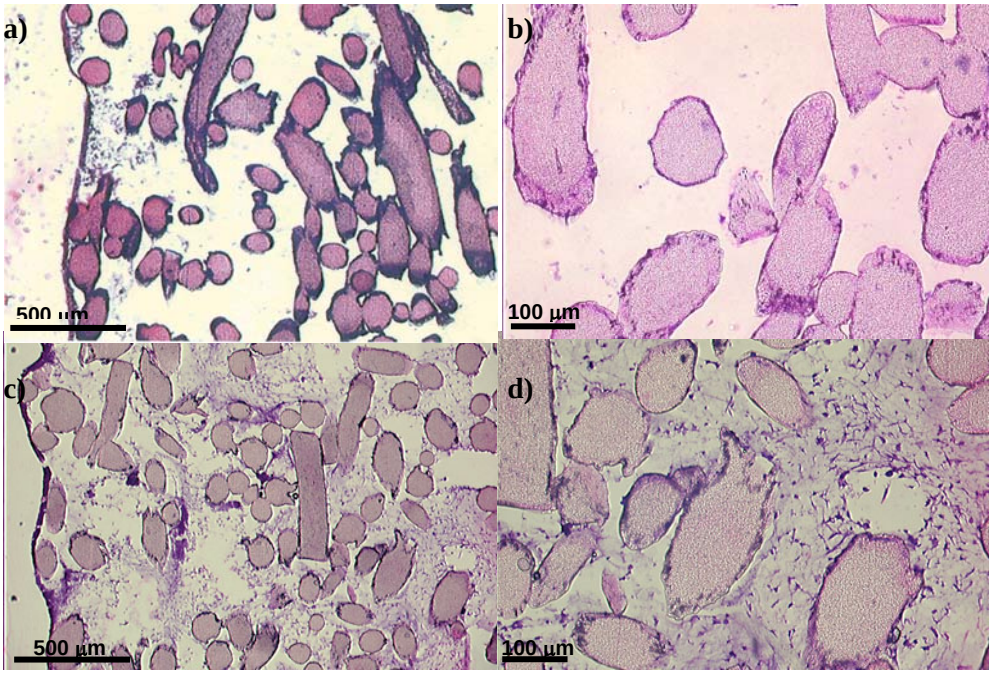

Figure V.7. Histological sections of SPCL fiber meshes cultured for 15 days and stained with hematoxylin and eosin, at different magnifications, in static (a,b) and flow perfusion culture (c,d).

3.6.2. Von Kossa staining

Figures V.8a through V.8c showed no mineral deposition on scaffolds cultured under static conditions. However, Von Kossa stained sections revealed that the mineralized tissue was distributed throughout the scaffolds cultured under flow perfusion (Figures V.8d through V.8f) in agreement with the histological observations and the calcium deposition measurements.

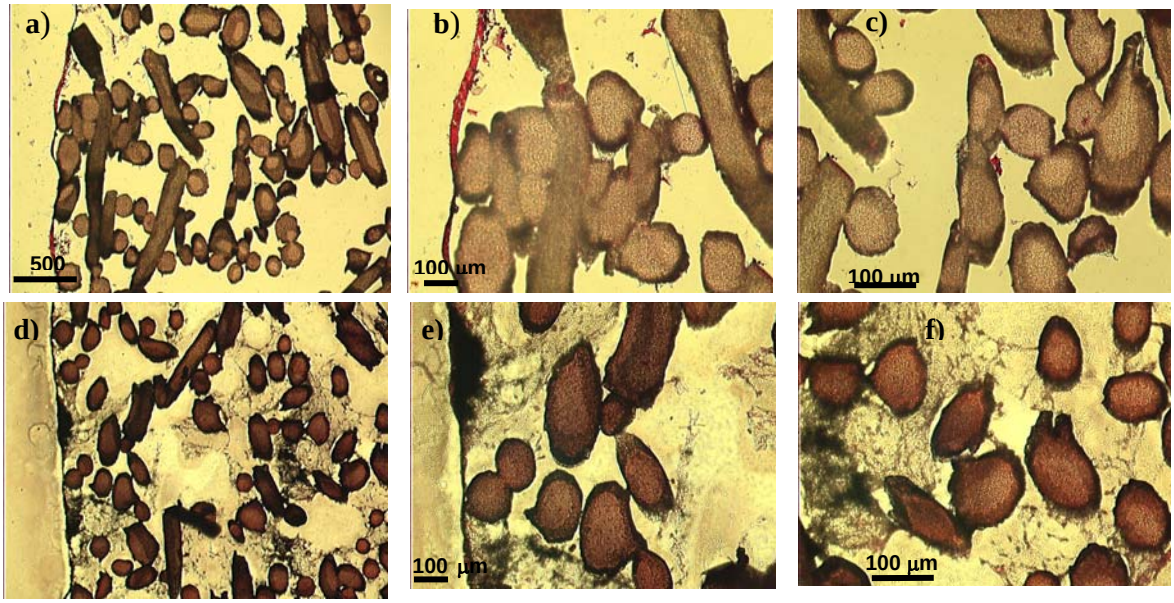

Figure V.8. Von Kossa stained histological sections of SPCL fiber meshes cultured for 15 days, at different magnifications, under static (a,b,c) and flow perfusion conditions (d,e,f).

3.6.3. Tetracycline fluorescence

In Figure V.9a the formation of a mineral layer on the surface of the scaffold is apparent. It is also possible to identify several regions in the interior of the scaffolds where mineral deposition has occurred. Once again, no mineral deposition is observed on the scaffolds cultured under static conditions (Figure V.9b). These results, together with the analysis of Von Kossa staining and calcium deposition, suggest that flow perfusion culture enhances the osteogenic differentiation of marrow stromal cells and improves their distribution in three-dimensional starch-based scaffolds by possibly improving nutrient delivery in the interior of the scaffolds or by stimulating the seeded cells by exposing them to fluid shear forces.

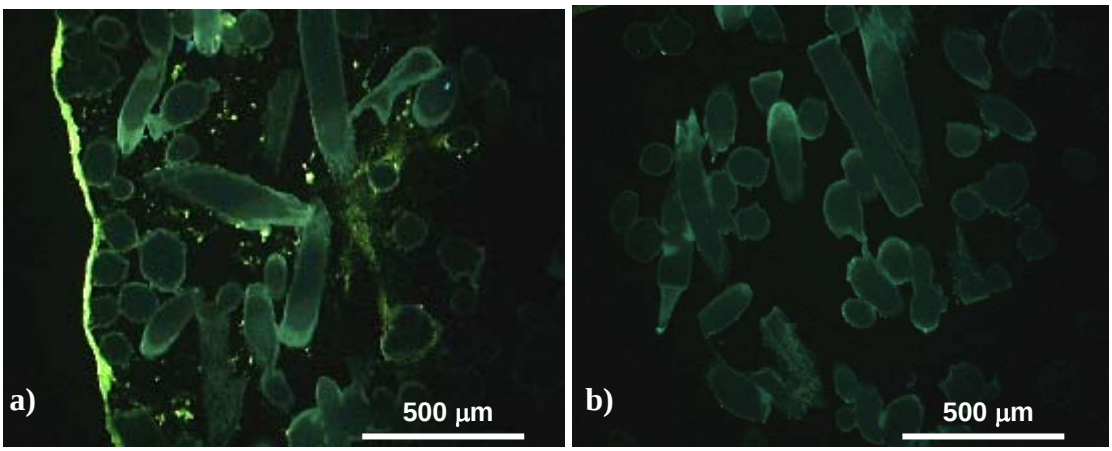

Figure V.9. Sections of SPCL fiber meshes cultured for 15 days observed under fluorescent light, showing the tetracycline labelling of mineral in (a) flow perfusion and (b) static culture conditions.

3.7. Scanning Electron Microscopy (SEM)

Figure V.10 shows SEM micrographs depicting the top (where the cells were seeded) and bottom surface of SPCL fiber meshes cultured for 15 days under flow and static conditions, respectively. The scaffolds cultured under flow perfusion conditions were completely covered by a dense matrix coating on both bottom and top surfaces, which suggests that cells were able to migrate throughout the scaffold and fill the entire construct. In contrast, the top surface of the scaffolds cultured under static conditions exhibited a thin crusting layer of extracellular matrix, and on the bottom surface it is only possible to observe the coating of some of the fibers. These observations reinforce the conclusion that flow perfusion enhances cell distribution in scaffolds cultured *in vitro*.

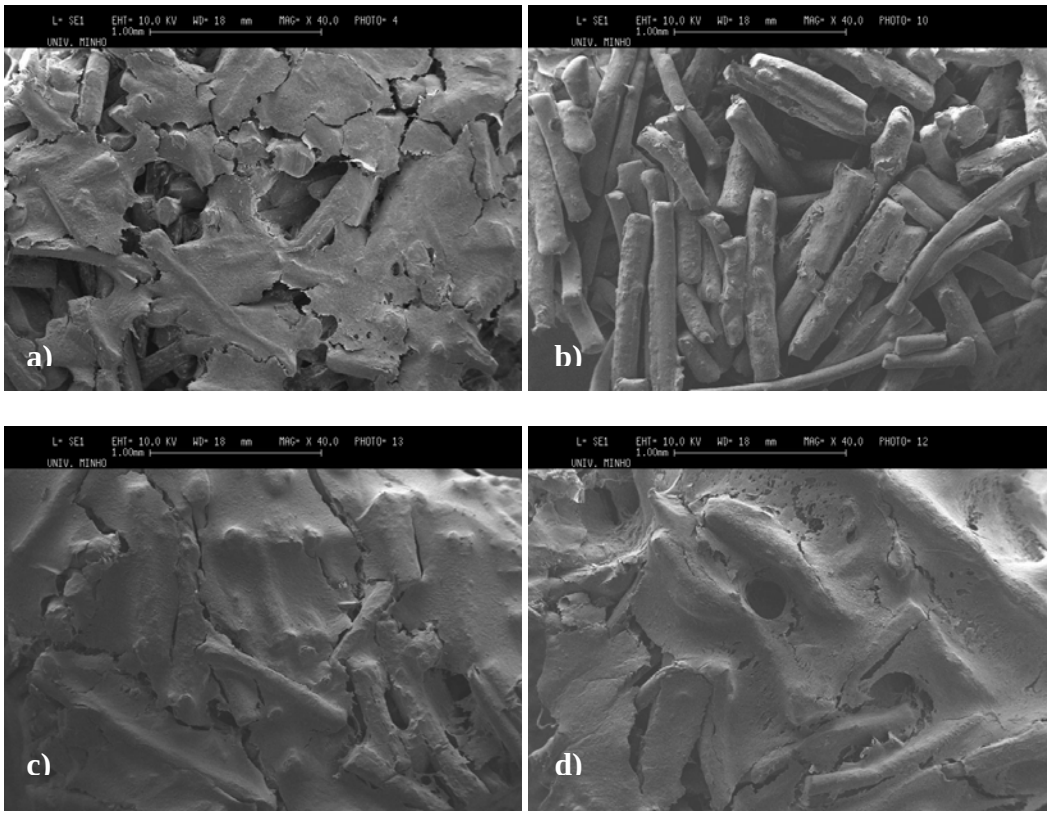

Figure V.10. SEM micrographs depicting the surface of SPCL fiber meshes cultured for 15 days: (a,c) top surface (surface where cells were seeded), (b,d) bottom surface (opposite to the surface where cells were seeded) of samples cultured under static (a,b) or flow perfusion conditions (c,d)

4. CONCLUSIONS

The two types of starch based scaffolds selected for this study promoted the attachment and proliferation of rat bone marrow stromal cells. However, the SPCL fiber meshes showed increased cell proliferation due to the better interconnectivity of their porous structure. This study demonstrates the ability of the flow perfusion bioreactor to enhance the osteogenic differentiation and the homogeneous distribution of marrow stromal cells within starch-based polymer scaffolds. Accordingly, starch-based porous scaffolds seeded with mesenchymal stem cells and cultured under flow perfusion constitute a promising approach for the generation of osteoinductive bone tissue replacement constructs.

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Chapter VI

INFLUENCE OF THE POROSITY OF STARCH-BASED FIBER MESH SCAFFOLDS ON THE PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF BONE MARROW STROMAL CELLS CULTURED IN A FLOW PERFUSION BIOREACTOR

Chapter VI

Influence of the Porosity of Starch-based Fiber Mesh Scaffolds on the Proliferation and Osteogenic Differentiation of Bone Marrow Stromal Cells Cultured in a Flow Perfusion Bioreactor*

Abstract

This study aims to investigate the influence of the porosity of fiber mesh scaffolds obtained from of a blend of starch and poly(ε-caprolactone) on the proliferation and osteogenic differentiation of marrow stromal cells cultured under static and flow perfusion conditions. For this purpose, biodegradable scaffolds were fabricated by a fiber bonding method into mesh structures with two different porosities, namely 50 and 75%. These scaffolds were then seeded with marrow stromal cells harvested from Wistar rats and cultured in a flow perfusion bioreactor or in 6-well plates for up to 15 days. The increased scaffold porosity significantly enhanced proliferation of cells cultured under both static and flow perfusion conditions. The expression of alkaline phosphatase activity was higher in flow cultures, but only for cells cultured onto the higher porosity scaffolds. Calcium deposition patterns were similar for both scaffolds, showing a significant enhancement of calcium deposition on cell-scaffold constructs cultured under flow perfusion, as compared to static cultures. An enhancement of calcium deposition was observed in scaffolds with higher porosity but this difference was not statistically significant. Scanning electron microscopy observation showed the formation of pore-like structures in the higher porosity scaffolds. Fourier transformed infrared spectroscopy-attenuated total reflectance and thin-film X-ray diffraction analysis of the cell-scaffold constructs after 15 days of culture in a flow perfusion bioreactor revealed the presence of a mineralized matrix, similar to bone. These findings indicate that starch-based scaffolds in conjunction with fluid flow bioreactor culture enable the creation of culture environments with minimal diffusion constraints and the ability to provide mechanical stimulation to the marrow stromal cells, leading to enhancement of their differentiation towards the development of bone-like mineralized tissue. These results also demonstrate that scaffold structure, namely the porosity, influences the sequential development of osteoblastic cells and in combination with the culture conditions may affect the functionality of *in vitro* formed tissues.

*** This chapter is based on the following publication:**

ME Gomes, HL Holtorf , RL Reis, AG Mikos. *Influence of the Porosity of Starch-based Fiber Mesh Scaffolds on the Proliferation and Osteogenic Differentiation of Bone Marrow Stromal Cells Cultured in a Flow Perfusion Bioreactor* . Tissue Engineering (2004) submitted

1. INTRODUCTION

In bone tissue engineering strategies based on cell-scaffold constructs, the macro and microstructural properties of the scaffolds in conjunction with the cell culture environment may play a very important role in the proliferation and differentiation of cells into the desired phenotype and consequently, the performance of the construct after implantation.
Adequate porosity and surface area are widely recognized [1-4] as important parameters in the design of scaffolds to be used in the development of tissue engineered substitutes. Other architectural features such as pore morphology and interconnectivity between pores of the scaffolding materials are also suggested to be important for cell seeding, migration, growth, mass transport, gene expression and new tissue formation in three dimensions.[1,2,5] Scaffolds with fiber mesh structures, for example, typically exhibit large surface area for cell attachment and a high interconnectivity among pores that enables a better diffusion of nutrients, enhancing cell survival and growth.
Bone tissue formation by osteogenic cells, such as marrow stromal cells, is characterized by sequential events involving cell proliferation, expression of osteoblastic markers and synthesis, deposition and mineralization of a collagenous matrix.[6] These events are greatly affected by the type of scaffold material in which the cells are seeded[7-11] but also by the culture environment[8,9,12]
In traditional cell culture systems, i.e., static systems, the cells within the constructs receive nutrients only by diffusion from the surrounding media[8,12] In this case, even in highly interconnected porous scaffolds, it is typical to observe a high cell density on the exterior of the scaffold that may deplete the nutrient supply before they can diffuse to the interior of the scaffold.[8] Consequently, the cells that are able to migrate to the interior of the scaffold may ultimately become necrotic due to the inaccessibility to the nutrients and also to the difficulty of removing metabolic wastes.[4,8] Furthermore, it is well known that bone cells are sensitive to mechanical stimulation[13,14] a constant in their natural environment, and that the absence of stresses may therefore negatively affect their development *in vitro.*[13] These findings have motivated the development of enhanced culture systems, such as the flow perfusion bioreactor[8,15-17] which may simultaneously provide sufficient and continuous level of oxygen and nutrient supplies and appropriate mechanical stimulation on cells,[8,17,18] creating 3-D culture environments that may support the formation of 3-D bone-like tissue *in vitro*.
Previous studies[19] demonstrated that biodegradable starch based scaffolds were able to support attachment, proliferation and differentiation of bone marrow stromal cells. The present study analyzed the influence of the porosity of fiber meshes based on the biodegradable blend of starch with poly(ε-caprolactone) on the proliferation and

differentiation of seeded rat bone marrow stromal cells cultured in a flow perfusion bioreactor and in 6-well plates. In this paper, the following questions are addressed: 1) What is the role/influence of scaffold porosity in the sequential development of cell-scaffold constructs cultured under flow perfusion as compared to static culture? 2) Does the porosity of the scaffolds and/or culture conditions affect the structure and composition of the tissue formed *in vitro*?

2. MATERIALS AND METHODS

2.1. Scaffold preparation and characterization

Two different scaffolds based on SPCL (a 30/70 wt% blend of starch with poly(ε-caprolactone)) were prepared by a fiber bonding process consisting of cutting and sintering fibers with a diameter of about 180μm, obtained by melt-spinning. The different porosity of the fiber meshes was obtained using different weight/amounts of fibers. The porosity of the scaffolds was determined by microcomputerized tomography (μCT) (ScanCo Medical μCT 80, Bassersdorf, Switzerland) at a resolution of 10 μm, and using at least 3 samples per group (of different porosity). The morphology of the porous structure was further characterized using a scanning electron microscope (Leica Cambridge S360, Leica Cambridge, UK), after sputter coating the samples with gold (Jeol JFC 1100, Jeol, USA).
All samples were cut into discs of approximately 8 mm diameter and 1.5 to 2 mm height and sterilized using ethylene oxide. Prior to cell seeding, the scaffolds were immersed in 30 ml of serum-free media in 50 ml tubes. Air was removed from their pores by generating vacuum with a 30 ml syringe equipped with an 18-gauge needle. The scaffolds were left in serum-free media overnight to allow swelling.

2.2. Isolation and expansion of rat bone marrow stromal cells

Rat bone marrow stromal cells were obtained from the femora and tibiae of male Wistar rats with weights ranging from 125 to 150 g (Harlan, USA). The isolation and culturing procedures of the rat bone marrow stromal cells were previously described.[20] Briefly, femora and tibiae were removed and washed in medium with a ten-fold higher concentration of antibiotics than normal culture medium. The epiphyses were cut off and the diaphyses flushed with 5 ml of complete media. The bone marrow obtained from all the rats was pooled and plated in 80cm^2 flasks. The cells were cultured at 37^{0}C in a humidified atmosphere with 5% CO_2 and the culture medium during the entire experimental period consisted of minimum essential medium (α-MEM Eagle, Sigma, USA), supplemented with

10% FCS (Fetal Calf Serum, Gemini, USA), 50 μg/ml ascorbic acid (Sigma, Chemical Co., St. Louis, MO, USA), 50 μg/ml gentamycin, 100 μg/ml ampicillin, 0.3 μg/ml fungizone, 10 mM β-glycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma). The culture medium was refreshed after 24h and thereafter every 2 days until day 6.

2.3. Cell seeding on starch-based scaffolds

At near confluence, after 6 days of primary culture, the adherent cells were enzymatically released using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA, Sigma), concentrated by centrifugation at 400g for 5 min and resuspended in complete media. Subsequently, the scaffolds (n=12 for flow and n=12 for static culture, for each scaffold type) were inserted into cassettes that were placed in 6-well plates. Each scaffold was then seeded with 300 μl of a cell suspension containing $5x10^5$ cells and incubated for 2 hours. Then 10 ml of complete media was added to each well. The seeded scaffolds were further incubated overnight to allow for cell attachment. The following day, seeded scaffolds were placed into fresh 6-well plates for static culture conditions or into the flow perfusion bioreactor and cultured in complete media for 7 and 15 days (6 scaffolds per culture condition and per culture time).

2.4. Cell culture: The flow perfusion culture system

In this study a flow perfusion bioreactor was used which is described in detail elsewhere.[15,16] Briefly, this bioreactor consists of 6 flow chambers, each one containing a cassette in which the scaffold is press-fitted. Gas permeable silicon tubing connects each flow chamber with a peristaltic pump and a medium reservoir. Each chamber has its own independent pumping circuit, but all pumps draw media from a common reservoir. For these experiments, culture media was pumped continuously at a flow rate of 1 ml/min through the cell/scaffold construct cassette/housing unit and re-circulated back to the reservoir. The total volume of medium in the flow system was 210 ml. In the static culture, 10 ml was added to each scaffold. In both culture systems, media was changed every 3 days. The entire flow perfusion bioreactor was maintained in an environment of 37°C with 5% CO_2.

At the end of each culture period, the cell/scaffold constructs were rinsed with phosphate buffered saline (PBS) and stored at -70°C in 10 ml tubes containing 1.4 ml of distilled, deionized water for DNA, calcium, and alkaline phosphatase (ALP) analysis. Before performing the assays, the samples were allowed to thaw at room temperature and then sonicated for about 15 min. Simultaneously, at each time point, two scaffolds from each

group were retrieved and fixed in a solution of 2.5% gluteraldehyde for SEM analysis and FTIR-ATR and TF-XRD.

2.5. Characterization of cultured scaffolds

2.5.1. Cellularity of scaffolds

The DNA content of each scaffold was measured using a PicoGreen DNA Quantification Kit (Molecular Probes). A description of the assay can be found elsewhere.[16] The cellularity of each scaffold was then calculated by correlation with the DNA of a known amount of marrow stromal cells.

2.5.2. Alkaline phosphatase activity

The alkaline phosphatase activity was measured using a colorimetric endpoint assay which measures the conversion of p-nitrophenyl phosphate to p-nitrophenol by the enzyme alkaline phosphatase[16] (Sigma Diagnostic Kit #104).

2.5.3. Calcium content of scaffolds

Cell/scaffold constructs were incubated overnight in 1N acetic acid to dissolve the deposited calcium. The calcium content was then measured using a colorimetric endpoint assay which measures the amount of calcium-cresolphthalein complexone formed when cresolphthalein complexone binds to free calcium in an alkaline solution (Sigma Diagnostic Kit #587). The amount of deposited calcium was expressed as mg of Ca^{2+} equivalents per scaffold.[16]

2.5.4. Scanning electron microscopy (SEM)

For SEM analysis the samples were fixed in a solution of 2.5% glutaraldehyde (in PBS), dehydrated in a gradient series of ethanol solutions, dried with tetramethylsilane, and sputter coated with gold (Jeol JFC 1100, Jeol, USA). Samples were then observed using a scanning electron microscope (Leica Cambridge S360, Leica, Cambridge, UK).

2.5.5. Fourier transformed infrared spectroscopy with attenuated total reflectance (FTIR-ATR)

The composition of the cell-scaffolds constructs cultured for 15 days in the perfusion bioreactor was analyzed by Fourier Transformed Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR). The samples were fixed in a solution of 2.5% gluteraldehyde and dried at room temperature. All spectra were recorded using at least 64

scans and 2cm^{-1} resolution in a FTIR spectrophotometer (Perkin-Elmer 1600 Series, USA) with a single reflection ATR system (MKII Golden Gate^{TM}, Specac, UK). Results were compared to cell-free scaffolds, which were kept under the same conditions as the static cultures and received the same treatment before performing the analysis, in order to exclude the contributions from the culture medium.

2.5.6. Thin-film X-ray diffraction (TF-XRD)

Thin-film X-ray diffraction (TF-XRD, Philips X'Pert MPD, The Netherlands) was used to identify any mineral phase present in cell-scaffolds constructs cultured in the perfusion bioreactor for 15 days. As for the FTIR-ATR analysis, the samples were dried at room temperature after being fixed in a solution of 2.5% gluteraldehyde. The data collection was performed by the 2θ scan method with 1º as incident beam angle using Cu$K\alpha$ X-ray line and a scan speed of 0.05º/min in 2θ. Again, the results were compared to cell-free scaffolds, which were kept in the same conditions and submitted to same treatment as samples resulting from static cultures.

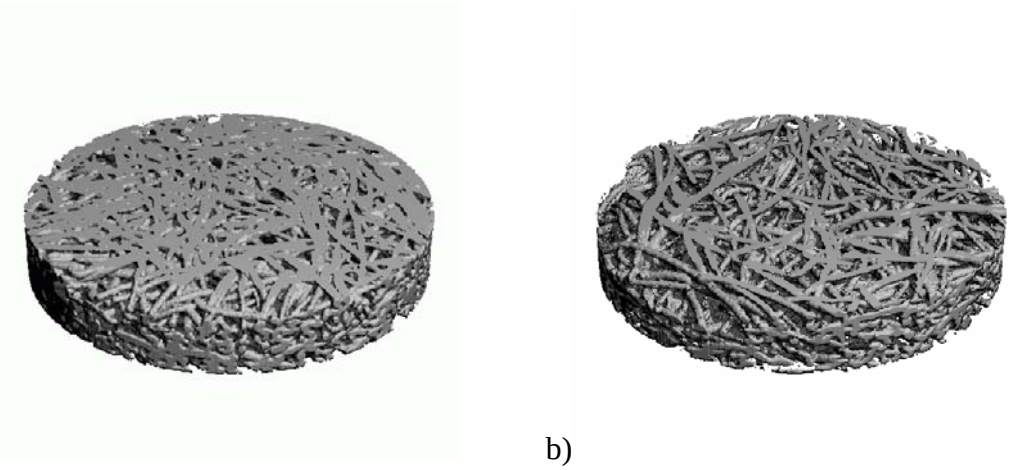
2.5.7. Statistics

Results from DNA, ALP and calcium assays are presented as means $\pm$ standard deviation for n=4. Multiple pairwise comparisons were performed using the Tukey-Kramer method with a significance level of 95%.

3. RESULTS AND DISCUSSION

3.1. Characterization of scaffold porosity

The two types of SPCL scaffolds produced for this study, exhibited a typical fiber-mesh structure (figure VI.1), and presented a porosity of 50,5±1,9 (figure 1a) and 74,5±1,2 (figure 1b), respectively, as determined by μCT analysis. The two scaffolds will be referred in this paper as 50% and 75% scaffolds, respectively.



a) b)

Figure VI.1. Representative μCT scans of (a) 50% porous SPCL based scaffold and (b) 75% porous SPCL based scaffold.

3.2. DNA analysis

The number of cells per scaffold (calculated from DNA measurements) at the end of each culture period studied is represented in figure VI.2. For both scaffolds (of different porosity) and for both culture conditions it is possible to distinguish a first period of active proliferation, correspondent to the first week of culture. This is followed by a period in which it is typical to observe the decrease of proliferation reflected by the decline in DNA synthesis, normally associated with matrix maturation and late stage differentiation of osteoblastic cells.[6] This behavior has been observed previously in marrow stromal cells seeded both into starch based scaffolds[19] and into titanium fiber meshes,[16,21] cultured under similar conditions.

The cellularity of SPCL scaffolds with 75% porosity after 7 and 15 days of culture was significantly higher ($p<0.05$) than the cellularity of scaffolds with 50% porosity cultured under the same conditions (static or flow perfusion conditions) for the same period of culture. These results suggest that higher porosity provides more space for cell proliferation but may also enhance diffusion of nutrients and facilitate metabolic waste removal that enables cell survival within the scaffolds.

Both 50% and 75% scaffolds cultured under flow perfusion show significantly higher ($p<0.05$) cellularity than the same type of scaffolds cultured under static conditions, for both culture periods studied. In a previous study,[19] in which the flow rate during perfusion culture was lower, it was demonstrated histologically that flow perfusion enables a better distribution of cells and matrix within the constructs due to the better diffusion of nutrients provided by this culture system. The present study is in accordance with this result. In addition, the comparison of the cellularity of 75% porous scaffolds with the previous

results[19] obtained for the same type of scaffold, demonstrates that increasing flow rate leads to enhanced cell proliferation, as was previously shown for titanium fiber meshes cultured under similar conditions.[16]

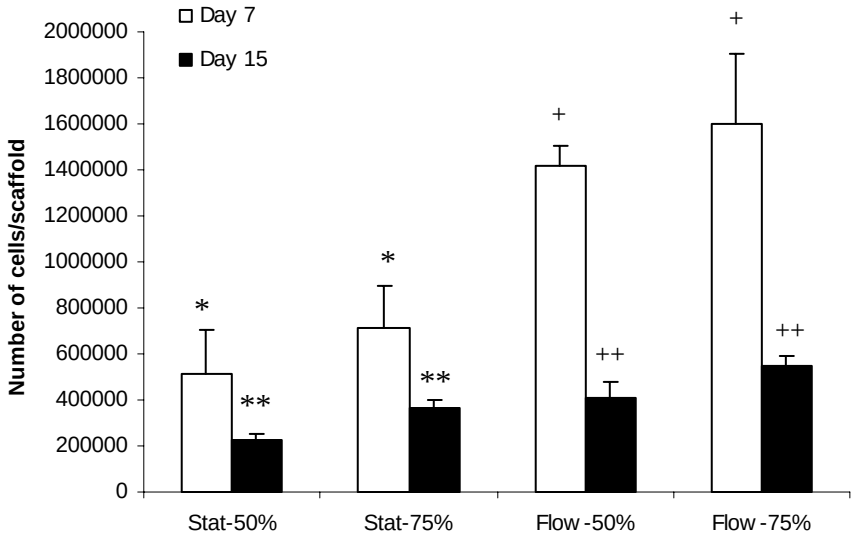


Figure VI.2. Number of cells on SPCL fiber meshes 75% porous and 50% porous after 7 and 15 days of culture under static and flow perfusion culture.

Error bars represent means ± standard deviation for n = 4.

The results assigned with different symbols are statistically different ($p<0.05$), indicating: (*) the higher cellularity in 75% porous scaffolds as compared to 50% in static cultures after 7 days of culturing;

(**) the higher cellularity in 75% porous scaffolds as compared to 50% in static cultures after 15 days of culturing;

(+) the higher cellularity in 75% porous scaffolds as compared to 50% in bioreactor cultures after 7 days of culturing;

(++) the higher cellularity in 75% porous scaffolds as compared to 50% in bioreactor cultures after 15 days of culturing.

The cellularity is also significantly higher in the bioreactor cultures, as compared to static cultures on both scaffolds, but this is not represented in order to simplify the data displayed.

3.3. Scanning electron microscopy

SEM micrographs obtained from the top surface (where cells were seeded) and the bottom surface of 50% and 75% porous scaffolds after 15 days of culture in the flow perfusion bioreactor (figure VI.3), show the presence of a dense cell matrix filling the constructs. However, the bottom surface of the 50% porous scaffolds is not completely covered with the dense cell layer observed on the bottom surface of the 75% porous scaffolds. This means that in 50% scaffolds the lower porosity hinders the penetration and distribution of

cells throughout the entire construct, as compared to 75% scaffolds. This observation is in agreement with the results obtained from DNA measurements, which indicate a higher cell number present in the 75% porous meshes. Additionally, in the higher porosity scaffolds the flow perfusion induced *de novo* tissue modeling with the formation of pore-like structures. The same is not observed in the 50% porous scaffolds. This result suggests the importance of the macrostructure, namely the porosity, not only on the amount of cells and matrix formed within the construct, but also on the structure of new tissue developed by tissue engineering approaches that involves an *in vitro* culturing stage prior to the implantation of the construct.

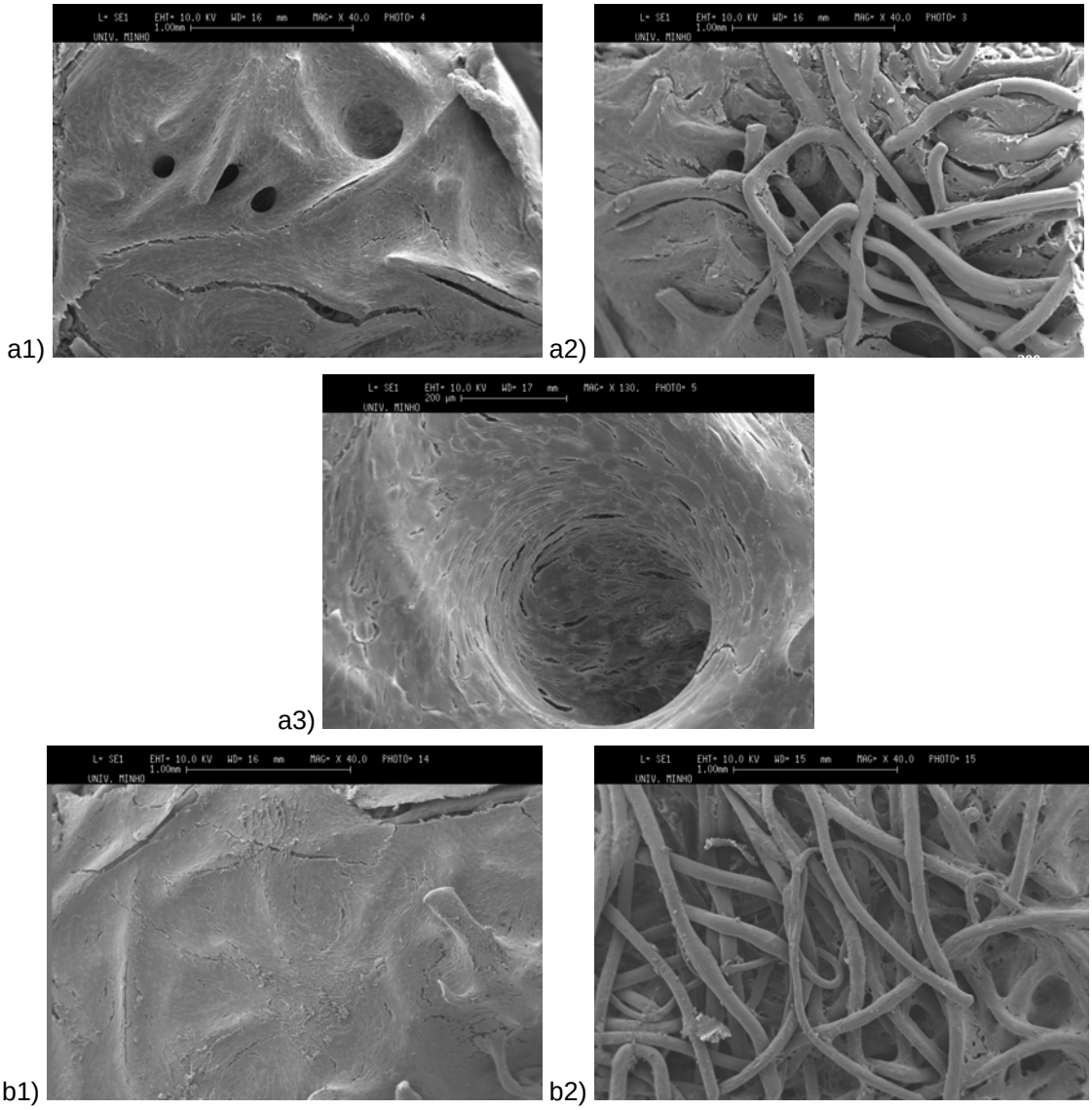


Figure VI.3. SEM micrographs depicting the surface of SPCL fiber meshes with a) 75% porosity and b) 50% porosity - cultured for 15 days in the flow perfusion bioreactor: a1 and b1) top surface (surface where cells were seeded) and a2 and b2) bottom surface (opposite to the surface where cells were seeded) of samples; a3) represents a magnification of a1, showing in detail the pore like structure formed in 75% scaffolds.

3.4. Alkaline phosphatase analysis

Figure VI.4 depicts the normalized ALP activity of marrow stromal cells seeded onto scaffolds with different porosity, after 7 and 15 days of culture under static and flow perfusion conditions. Alkaline phosphatase is a protein associated with bone cell phenotype, and its expression is found to increase significantly upon the stage of active proliferation of osteoblastic cells.[6] During the stage of matrix maturation, the extracellular matrix undergoes several modifications in composition and organization that ultimately lead to its mineralization. At this point, every cell becomes alkaline phosphatase positive.[6] In the present study, a significant increase of the ALP activity from day 7 to day 15 of culture was observed for all samples cultured under static and flow perfusion conditions, in agreement with the patterns described in the literature.[6] In this case, the scaffold porosity does not have a strong influence in this marker of osteoblastic differentiation, as no significant difference was found between the ALP levels correspondent to samples of different porosity. However, comparing the ALP activity of cells on 75% meshes cultured in static and flow perfusion systems, significantly higher expression of ALP was found in perfusion cultures for the same period of culture. The enhanced ALP of perfusion cultures has been observed before for titanium fiber meshes[21], but finding this difference only in the 75% scaffolds suggests that porosity (combined with flow culture conditions) has an important role in the sequential development of osteogenic cells. Additionally, ALP is known to be a transient marker of osteoblastic differentiation, being up-regulated initially and down-regulated as differentiation progresses. Because of this phenomenon and because samples were analyzed at only two time points, the presence or lack of statistical difference in ALP levels between meshes or time points may be a result of ALP activity peaking in between time points. This also explains why it is possible to see a statistical difference in end-stage markers such as calcium deposition when none was seen for the earlier stage marker ALP.

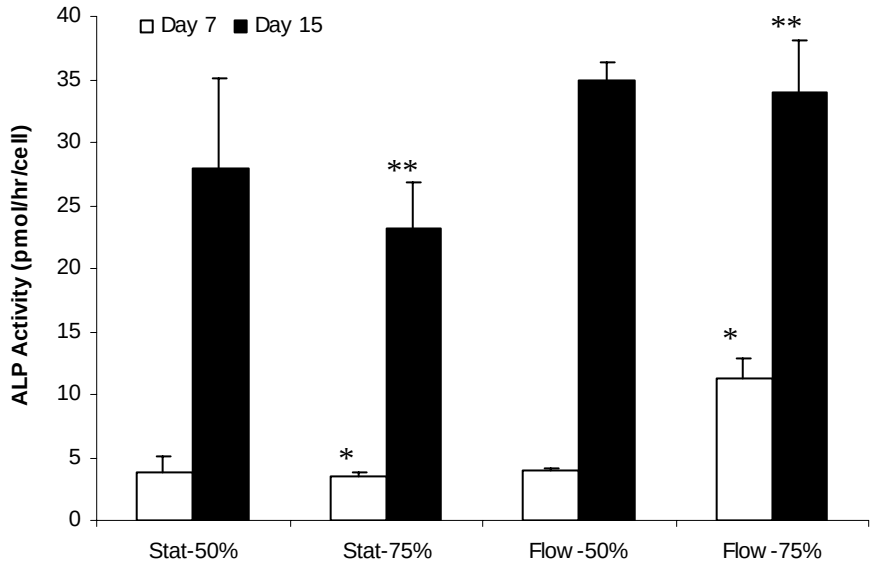


Figure VI.4. Normalized ALP activity of marrow stromal cells after 7 and 15 days of culture on SPCL fiber meshes 75% porous and 50% porous.

Error bars represent means ± standard deviation for n = 4.

Asterisk (* and **) assign statistically different results ($p<0.05$), indicating:

(*) the significantly higher level of ALP activity observed for 75% scaffolds cultured under flow perfusion, compared to static cultures for 7 days of culture.

(**) the significantly higher level of ALP activity observed for 75% scaffolds cultured under flow perfusion, compared to static cultures for 15 days of culture.

3.5. Calcium deposition

The calcium deposition per scaffold, in 50% and 75% scaffolds cultured under static and flow perfusion conditions after 7 and 15 days, is graphically represented in figure 5. The mineral accumulation is a consequence of the progression of pre-osteoblastic cells through the proliferation and matrix maturation stages of differentiation and it is an essential step for the further up-regulation or expression of genes responsible for the mineralization of the extracellular matrix.[6] In this study, calcium measurements show that by the end of the first week of culture there is practically no calcium deposition for both scaffolds and for both culture conditions. At the end of the second week, however, a dramatic increase in calcium deposition was observed in the scaffolds cultured in the flow perfusion bioreactor. In fact, the calcium deposited on the scaffolds cultured under flow perfusion conditions (both for 75% and 50% porous scaffolds) after 15 days of culture was significantly higher ($p<0.05$) than calcium deposited on scaffolds cultured under static conditions, in agreement with previous results.[19]

Calcium deposition in 75% porous scaffolds was higher than for 50% scaffolds, but the difference was not significant, although the shear forces experienced by cells are expected to increase in fiber meshes exhibiting lower porosity. These forces can be calculated assuming a cylindrical pore model approximation for the scaffold pore morphology.[22] The shear forces experienced by the cells seeded into 75% and 50% porous scaffolds were estimated to be on the order of 0.2 dyn/cm^2 and 0.3 dyn/cm^2, respectively. The higher calcium deposition reported for the scaffolds of higher porosity may be explained by the higher number of cells and cell density registered in these scaffolds (as demonstrated by the DNA analysis), which is a very important factor in mineralization. These results further support the importance of scaffold structure and culture conditions on the osteogenic differentiation of bone marrow stromal cells. The results obtained for the 75% scaffolds using a flow rate of 1ml/min are also significantly higher than those obtained in a previous study[19] for similar scaffolds cultured at lower flow rates (0.3 ml/min). This effect has been observed in other studies[16,21] and is associated with increased mechanical stimulation induced by the increased fluid shear stresses experienced by the cells cultured under higher flow rates.

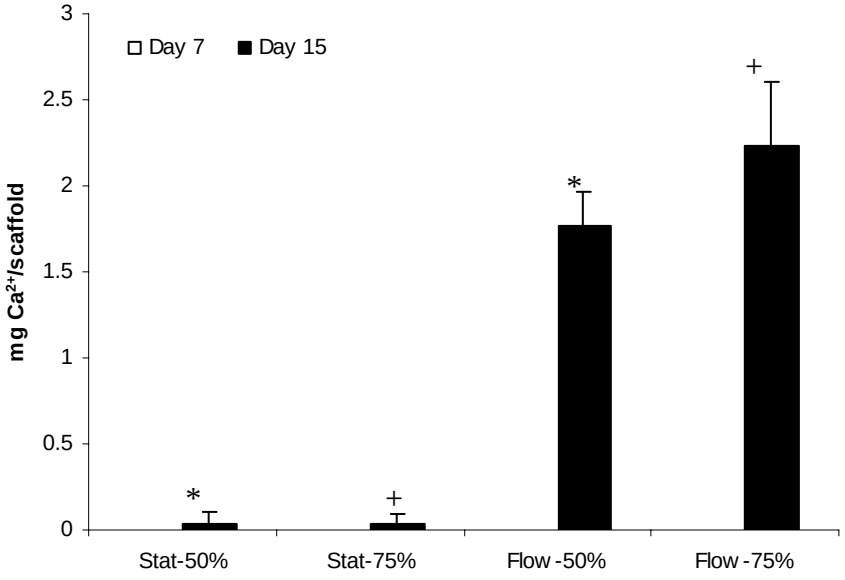


Figure VI.5. Calcium deposition on SPCL fiber meshes 75% porous and 50% porous after 7 and 15 days of culture under static and flow perfusion conditions.
Error bars represent means ± standard deviation for n = 4.
Symbols (* and +) assign statistically different results ($p<0.05$), indicating:
(*) the significantly higher calcium deposition after 15 days of culture in flow cultures as compared to static cultures of 50% porous scaffolds.
(+) the significantly higher calcium deposition after 15 days of culture in flow cultures as compared to static cultures of 75% porous scaffolds.

3.6. FTIR-ATR

To demonstrate the clinical feasibility of tissue engineered bone and to sufficiently match the intrinsic properties of autogenous bone-graft material, rapid mineralization of osteoid tissue grown *in vitro* must be achieved.[12] Nevertheless, the analysis of mineral deposition occurring in *in vitro* cultures is frequently based on Von Kossa or Alizarin red staining or by calcium uptake.[23] However, the results arising from these analyses can be misinterpreted, as the matrix is known to uptake calcium independently of mineral deposition. Therefore, these methods should be complemented with diffraction or spectroscopy methods.[23]

In this study, FTIR-ATR spectra were obtained from 75 and 50% porous meshes cultured for 15 days in the perfusion bioreactor (Figure VI.6). These spectra showed very intense amide bands at about 1634 cm^{-1} (amide I) and 1525 cm^{-1} (amide II), that can be assigned to the protein matrix formed.

The FTIR-ATR spectrum also displays reflectance peaks associated with the phosphate group in carbonated apatite (group bands at 1041 cm^{-1} and 562 cm^{-1}), clearly suggesting the presence of mineralized extracellular matrix.

The spectra collected for both 50% and 75% porous scaffolds were identical.

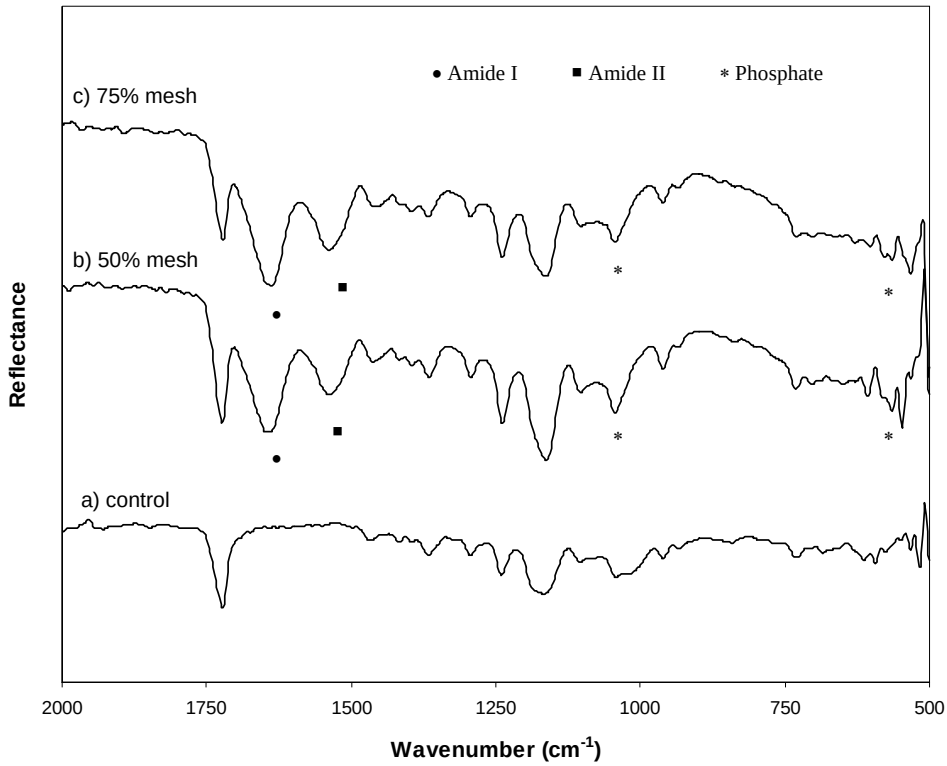


Figure VI.6. FTIR-ATR spectra of a) Control – cell-free scaffold kept in the same conditions as for static cultures and treated with the same fixative solution. b) 50% porous scaffolds cultured in the flow perfusion bioreactor for 15 days c) 75% porous scaffolds cultured in the flow perfusion bioreactor for 15 days.

3.7. TF-XRD

Figure 7 shows the TF-XRD patterns of cell/scaffold constructs of different porosities after 15 days of culture in the flow perfusion bioreactor, using cell-free scaffolds as controls. In these diffraction patterns, several characteristic peaks of hydroxyapatite were evidenced, that were confirmed by comparing with XRD of standard hydroxyapatite (JCPDS 9-432). The apatite formed seems to be mainly amorphous, as it is observed for minerals occurring in the presence of proteins, such as apatite in bone.[24]

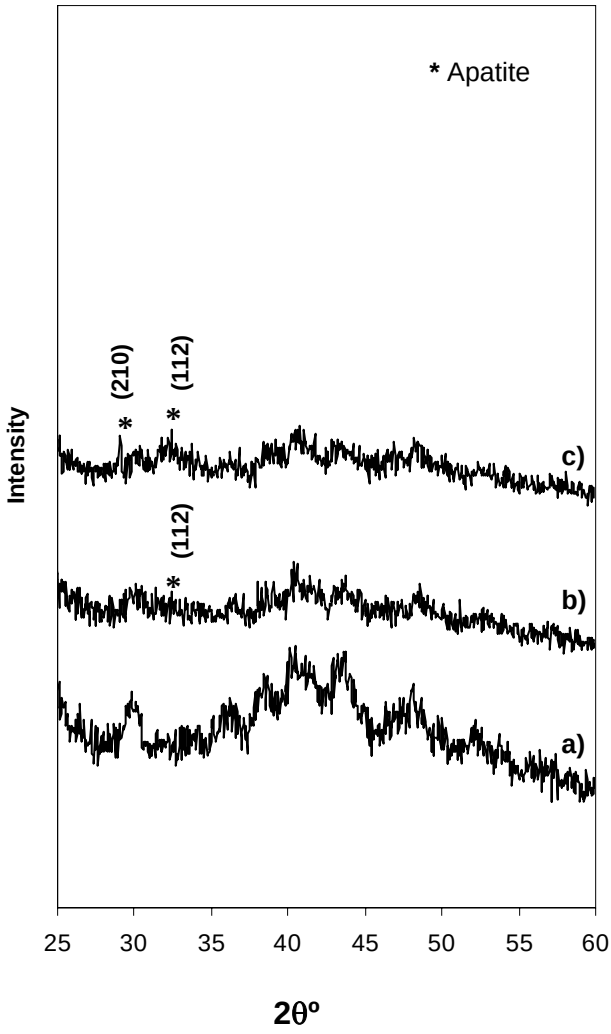


Figure VI.7. TF-XRD patterns of the a) Control – cell-free scaffold, kept in the same conditions as for static cultures and treated with the same fixative solution. b) 50% porous scaffolds cultured in the flow perfusion bioreactor for 15 days c) 75% porous scaffolds cultured in the flow perfusion bioreactor for 15 days.

The patterns correspondent to 50% scaffolds, are similar to those for the 75% scaffolds. However, it was found that the diffraction peaks of the apatite phase exhibited lower

intensity in 50% scaffolds and one of the peaks, correspondent to the plane (210), assigned in the patterns of the 75% porous scaffold, is not evidenced in the patterns correspondent to 50% porous scaffolds. This might be a further indication of the influence of porosity in the development of the mineralized extracellular matrix by marrow stromal cells cultured under flow perfusion conditions. These results, together with the FTIR-ATR spectra, confirm that the mineral formed by marrow stromal cells seeded in SPCL fiber meshes and cultured under flow perfusion conditions is, in fact, a carbonated apatite mineral similar to the major mineral component of bone.[25]

4. CONCLUSIONS

This study showed that biodegradable starch-based fiber mesh scaffolds in conjunction with fluid flow bioreactor culture enable the creation of culture environments with minimal diffusional constraints and the ability to provide mechanical stimulation to seeded marrow stromal cells, leading to enhancement of their differentiation towards the development of a bone-like extracellular matrix and its mineralization, forming a carbonated apatite mineral similar to the major mineral component of bone.

This study demonstrated that increased scaffold porosity significantly enhances the proliferation of marrow stromal cells cultured under static and flow perfusion conditions and influences the sequential development of the seeded cells. Furthermore, the flow perfusion induces *de novo* tissue modeling with the formation of pore-like structures in the scaffolds with higher porosity, demonstrating that this structural aspect of scaffolding materials, in combination with the culture environment determines, to a great extent, the structure and possibly the functionality of bone-like tissue substitutes formed *in vitro*.

In summary, the culturing of highly porous starch based fiber mesh scaffolds, seeded with marrow stromal cells, under flow perfusion conditions may allow for the development of adequate *in vitro* engineered substitutes for the repair of bone tissue.

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CHAPTER VII

IN VITRO EXPRESSION OF BONE GROWTH FACTORS BY MARROW STROMAL CELLS CULTURED IN STARCH/POLY(ε-CAPROLACTONE) SCAFFOLDS USING A FLOW PERFUSION BIOREACTOR

Chapter VII

In Vitro Expression of Bone Growth Factors by Marrow Stromal Cells Cultured in Starch/Poly(ε-Caprolactone) Scaffolds Using a Flow Perfusion Bioreactor *

Abstract

Tissue engineering strategies aim at controlling the behaviour of individual cells to stimulate tissue formation. This control is achieved by mimicking signals that manage natural tissue development or repair. Flow perfusion bioreactors that create culture environments with minimal diffusion constraints and provide cells with mechanical stimulation, may closely resemble *in vivo* conditions for bone formation. Therefore, these culturing systems, in conjunction with an appropriate scaffold and cell type, may provide significant insight towards the development of *in vitro* tissue engineering models leading to improved strategies for the construction of bone tissue substitutes.

The objective of this study was to investigate the *in vitro* expression of several bone growth factors that are usually associated with bone formation *in vivo* by culturing rat bone marrow stromal cells seeded onto starch-based biodegradable fiber meshes in a flow perfusion bioreactor. The expression of several bone related growth factors, namely transforming growth factor-β1 (TGF-β1), platelet derived growth factor-A (PDGF-A), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2), was observed on two different time points in scaffolds cultured under perfusion conditions at different flow rates, using an immunohistochemistry technique. The results show the presence of regions positively stained for all the growth factors analysed, except for PDGF-A. Furthermore, the images obtained from the positively stained sections suggest an increase in the immunohistochemically stained area with increasing flow rate and also a trend of enhanced growth factor expression with culturing time. These observations demonstrate that flow perfusion augments the functionality of scaffold/cell constructs grown *in vitro* as it combines both biological and mechanical factors to enhance cell differentiation and cell organization within the construct. This study also shows that flow perfusion bioreactor culture of marrow stromal cells combined with the use of appropriate biodegradable fiber meshes may constitute a useful model to study bone formation and assess bone tissue engineering strategies *in vitro*.

*** This chapter is based on the following publication:**

ME Gomes, CM Bossano, CM Johnston, RL Reis, AG Mikos. In Vitro Expression of Bone Growth Factors by MSCs Cultured on Starch/poly(ε-caprolactone) Scaffolds Using a Flow Perfusion Bioreactor. Tissue Engineering (2004) *submitted*

1. INTRODUCTION

Bone tissue engineering approaches based on biodegradable scaffolds seeded with cells and cultured *in vitro* prior to implantation, aim at creating *in vitro* environments that mimic, to a certain extent, the biochemical and mechanical signals responsible for natural bone development and repair. Therefore, in order to design successful strategies, that enable the development of functional bone-like tissue substitutes, it is important to understand the mechanisms of cell growth and differentiation involved in the formation of bone tissue.
Although techniques for studying bone formation *in vivo* have been well developed for many years, there are not adequate *in vitro* models available. This is due in part to the complexity of the bone formation process. It is unlikely that osteoblastic cells in culture will express and secrete all of the proteins necessary for the formation of normally mineralized bone *in vitro* [1] as it is difficult to model all the complexities of the *in vivo* environment [2]. Nevertheless, the available data, most of which are based on observations of normal fetal rat calvarial osteoblasts in prolonged culture, suggest that the process of bone formation depends on the sequential expression and interaction of a number of growth factors [1,3].

Growth factors are proteins secreted by cells that act on an appropriate target cell or cells to carry out a specific action. They function as part of a vast cellular communications network that influences critical functions such as cell division, matrix synthesis and tissue differentiation [4,5]. According to the type of action they produce, growth factors can be classified as i) autocrine, when the growth factor influences the cell of its origin or other cells identical in phenotype to that cell (eg., a growth factor produced by an osteoblast influences the activity of another osteoblast), ii) paracrine, when the growth factor influences an adjacent or neighbouring cell that is different in phenotype from its cell of origin (e.g., growth factor produced by an osteoblast stimulates differentiation of an undifferentiated cell), and iii) endocrine, when the growth factor influences a cell that is different in phenotype from the cell of its origin and located at a remote anatomical site (e.g., a growth factor produced by neural tissue in the central nervous system stimulates osteoblast activity). Thus, a growth factor may affect multiple cell types and may induce an array of cellular functions in a variety of tissues [4,5].

The results of experimental studies have established that growth factors play an important role in bone and cartilage formation, fracture-healing and the repair of musculoskeletal tissues [4-8]. Among the most widely studied bone growth factors are transforming growth factor-β1 (TGF-β1), platelet derived growth factor (PDGF), bone morphogenetic proteins

(BMPs), fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) [4-9]. However, other growth factors have also been shown to have an effect on bone cells [10]. These include members of the epidermal growth factor (EGF) family, transforming growth factor-α (TGF-α) and vascular endothelial growth factor (VEGF) [10]. In fact, the total number of growth factors that can affect proliferation, differentiation and secretory functions of bone related cells is unknown, but the number increases continually as a result of new advanced techniques in protein biochemistry and molecular biology [5]. Therefore, assessment of bone growth factors expression may provide important information on the functionality of bone-like tissue substitutes developed by *in vitro* bone tissue engineering systems. Additionally it may provide clues for a better understanding of bone formation mechanisms.

In previous studies [11-13] we have used a flow perfusion bioreactor as a culturing system for rat bone marrow stromal cells seeded onto starch-based biodegradable scaffolds in order to evaluate the potential of this tissue engineering approach for the generation of osteoinductive bone tissue replacement constructs. The results show that biodegradable starch-based scaffolds in conjunction with fluid flow bioreactor culture enable the creation of culture environments with minimal diffusional constraints and the ability to provide mechanical stimulation to marrow stromal cells[11-13]. This leads to enhancement of their differentiation and development of a bone-like extracellular matrix consisting of a carbonated apatite mineral similar to the major mineral component of bone [11,12]. The design of this flow perfusion bioreactor enhances the distribution of nutrients because it allows the transport of medium through the interconnected pores of the scaffold. In addition, it offers a convenient way of providing mechanical stimulation to cells by means of fluid shear stress, which is particularly important in bone tissue engineering since bone cells are known to be stimulated by mechanical signals [14,15]. Furthermore, the magnitude of the shear stresses experienced by the cells can be varied by adjusting the medium flow rate through the system [16]. Therefore, the characteristics of the flow perfusion bioreactor in conjunction with scaffolds of suitable properties and porous structure may facilitate the *in vitro* development of tissue-like constructs for the regeneration of bone tissue defects.

The present study aims to investigate the *in vitro* expression of several bone growth factors that are usually associated with bone formation *in vivo* by culturing rat bone marrow stromal cells seeded onto SPCL (a blend of starch with poly(ε-caprolactone)) fiber meshes in a flow perfusion bioreactor at different flow rates and culture periods.
This research work describes the use of an immunohistochemical technique to assess the expression of several bone related growth factors by cells seeded onto a biodegradable

scaffold and cultured *in vitro,* using a specific bioreactor previously developed for bone tissue engineering applications. Specifically, this study was designed to obtain answers to the following questions: i) is it possible to use immunohistochemical techniques to analyze expression of bone growth factors on samples cultured *in vitro*? ii) are bone growth factors, usually found *in vivo*, expressed by marrow stromal cells cultured in a flow perfusion bioreactor iii) is the *in vitro* tissue engineering approach described suitable to be used as a model for studies on bone formation?

2. MATERIALS AND METHODS

2.1. Scaffold preparation

The starch-based polymer scaffold used in this study was based on SPCL (a blend of corn starch with poly(ε-caprolactone), 30/70 %wt) obtained by a fiber bonding process consisting of the spinning, cutting and sintering of fibers with a diameter of about 180μm. The porosity and porous structure of these scaffolds were characterized previously by microcomputed tomography (μCT) and scanning electron microscopy (SEM), showing that the scaffolds exhibit a typical interconnected fiber mesh structure, with a porosity of about 75%[13].

All samples were cut into discs of approximately 8 mm diameter and 1.5 to 2 mm height and sterilized using ethylene oxide. Prior to cell seeding, the scaffolds were immersed in 30 ml of serum-free medium in 50 ml tubes. Air was removed from their pores by generating vacuum with a 30 ml syringe equipped with an 18-gauge needle. The scaffolds were left in serum-free medium overnight to allow for swelling.

2.2. Isolation and expansion of rat bone marrow stromal cells

Rat bone marrow stromal cells (MSCs) were obtained from the femora and tibiae of male Wistar rats with weights ranging from 125 to 149 g (Harlan, USA). The isolation and culturing procedures of the rat bone marrow stromal cells were described in detail elsewhere[13]. Briefly, femora and tibiae were removed and washed in medium with an antibiotic concentration 10 times higher than in the complete culture medium used in the remaining experiments. The epiphyses were cut off and the diaphyses flushed with 5 ml of complete media (see bellow). The bone marrow obtained from all the rats was pooled and plated into 75cm^2 flasks. The cells were cultured at 37^{0}C in a humidified atmosphere with 5% CO_2 and the complete culture medium during the entire experimental period consisted of minimum essential medium (α-MEM Eagle, Sigma, St. Louis, MO, USA), supplemented

with 10% FCS (Fetal Calf Serum, Gemini, USA), 50 μg/ml ascorbic acid, 50 μg/ml gentamycin, 100 μg/ml ampicillin, 0.3 μg/ml fungizone, 10 mM β-glycerophosphate, and 10^{-8} M dexamethasone (Sigma). The culture medium was refreshed after 24h and thereafter each 2 days until day 6.

2.3. Cell seeding on starch-based scaffolds

At near confluence, after 6 days of primary culture, the adherent cells were enzymatically released using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA, Sigma), concentrated by centrifugation at 400g for 5 min and resuspended in complete medium. Subsequently, the scaffolds were inserted into flow system cassettes that were placed in 6-well plates. Each scaffold was then seeded with 300 μl of a cells suspension containing $5x10^5$ cells and incubated for 2 hours. Then 10 ml of media was added to each well and the seeded scaffolds were incubated overnight to allow further cell attachment. The following day, seeded scaffolds were placed into the flow perfusion bioreactor and cultured in complete media for 10 and 16 days (6 scaffolds per culture time). Seeded scaffolds, cultured in static conditions (6-well plates) were used as controls.

2.4. Cell culturing: The flow perfusion culture system

The flow perfusion bioreactor used in this study is described in detail elsewhere [16,17]. Briefly, this bioreactor consists of 6 flow chambers, each one containing a cassette in which the scaffold is press-fitted. Gas permeable silicon tubing connects each flow chamber with a peristaltic pump and a medium reservoir. Each chamber has its own independent pumping circuit, but all pumps draw media from a common reservoir. For these experiments, two bioreactor systems were used simultaneously, at two different flow rates, namely 1 ml/min and 0.3 ml/min. The total volume of medium continuously flowing in the system was 210 ml and the whole volume was changed every 3 days. The entire flow perfusion bioreactor was maintained in an environment of 37°C with 5% CO_2.

2.5. Immunohistochemistry analysis

2.5.1. Samples processing:

At the end of each culturing period, the cell-mesh constructs were removed from the bioreactors, rinsed with a phosphate buffer saline solution (PBS, 0.01M, pH 7.4) and fixed in a 10% formalin solution (Sigma). The constructs were then rinsed with PBS, cut in halves, embedded in optimal freezing temperature (O.C.T.) compound (Tissue-Tek, USA)

and frozen on dry ice. Serial sections (10 μm in thickness) were prepared and stored at –80ºC until staining.

2.5.2. Immunostaining procedure:

The sections were immunostained using previously established protocols [18] using the avidin-biotin immunoperoxidase staining technique which is based on the ability of egg-white glycoprotein avidin to non-immunologically bind four molecules of the vitamin biotin [19]. This technique uses 3 main reagents: the first is a primary antibody specific for the antigen to be localized; the second is antimouse/antigoat polyclonal antibody (secondary) which is covalently linked to a molecule of biotin and the third is a complex of peroxidase conjugated with biotin and avidin. The free sites on the avidin molecule allow binding to the biotin on the second antibody. The peroxidase enzyme, and therefore the original antigen, is identified with an appropriate chromogen [19].

The experimental staining procedure can be briefly described as follows: the sections were first incubated with a hydrogen peroxide solution to block against endogenous peroxidase activity and then incubated with normal serum to block against random secondary antibody binding. Afterwards, the sections were incubated with primary antibody for the antigen of interest and then incubated with the avidin-biotin secondary antibody system and with 3,3'-diaminobenzidin developing reagent (DAB, Vector Laboratories). As a last step, the sections were counterstained with hematoxylin and mounted. The negative staining controls consisted of sections incubated with 0.01M PBS instead of the primary antibody. A group of samples received conventional hematoxilin and eosin staining.

2.5.3. Primary antibodies:

For this study, the following antibodies were selected: anti-transforming growth factor-β1 (anti-TGF-β1, goat polyclonal antibody, sc-146-G), anti-platelet derived growth factor-A (anti-PDGF-A, mouse monoclonal antibody, sc-9974), anti-fibroblast growth factor-2 (anti-FGF-2, goat polyclonal antibody, sc-79-G), anti-vascular endothelial growth factor (anti-VEGF, mouse monoclonal antibody, sc-7269) and anti-bone morphogenetic protein-2 (anti-BMP-2, goat polyclonal antibody, sc-6895). All the antibodies were purchased from Santa Cruz Biotechnology (California, USA) and used at a concentration of 2μg per ml of 0.1 PBS. The diluted antibody solutions were used in the same day they were prepared. The mouse monoclonal antibodies were used in conjunction with an anti-mouse avidin-biotin complex ABC kit and the goat polyclonal antibodies were used in conjunction with an anti-goat ABC kit (both Vectastain Elite® ABC kit, Vector Laboratories).

2.5.4. Image acquisition:

All the stained sections were observed with an Eclipse E600 light microscope (Nikon, Melville, NY, USA) equipped with a CCD camera (Sony DXC-950P, NY, USA). For each stained section, 3 digital images were taken, corresponding to the opposite ends and center of the sample, as shown in figure VII.1 at a magnification of x4.

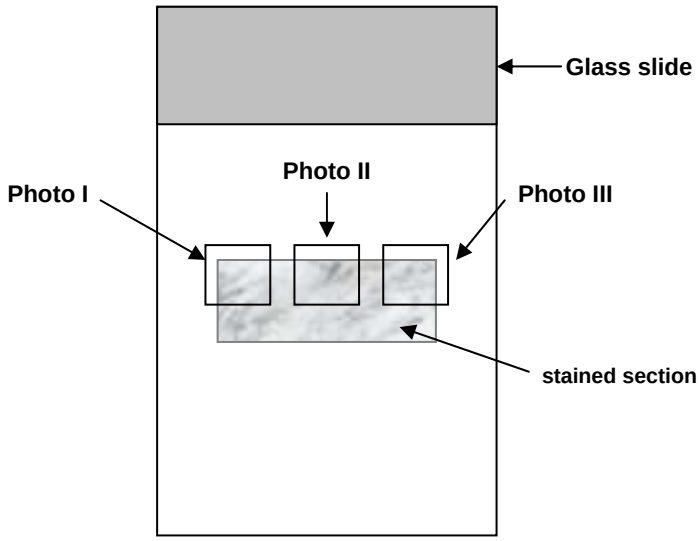

Figure VII.1. Schematic representation of image acquisition scheme used: for each stained section that was observed, 3 digital images were taken, corresponding to the opposite ends and center of the sample, as it is indicated.

3. RESULTS & DISCUSSION

Histological analysis of SPCL scaffold/cell constructs stained for H&E showed the presence of cells and matrix distributed throughout the interior of the three-dimensional starch-based scaffolds and also the formation of a thick surface layer of cells, as shown of figure VII.2. Furthermore, these images suggest an increase in the amount of cell and matrix with increasing flow rate, in agreement with previous studies[13,15,17] which have demonstrated that flow perfusion culture enhances the osteoblastic differentiation of marrow stromal cells (MSCs) and improves their distribution in the scaffolds in a dose-dependent manner by improving nutrient delivery to the interior of the scaffolds and stimulating the seeded cells by exposing them to fluid shear forces. As expected, an increase in the amount of cells and matrix with increasing culture time was observed.

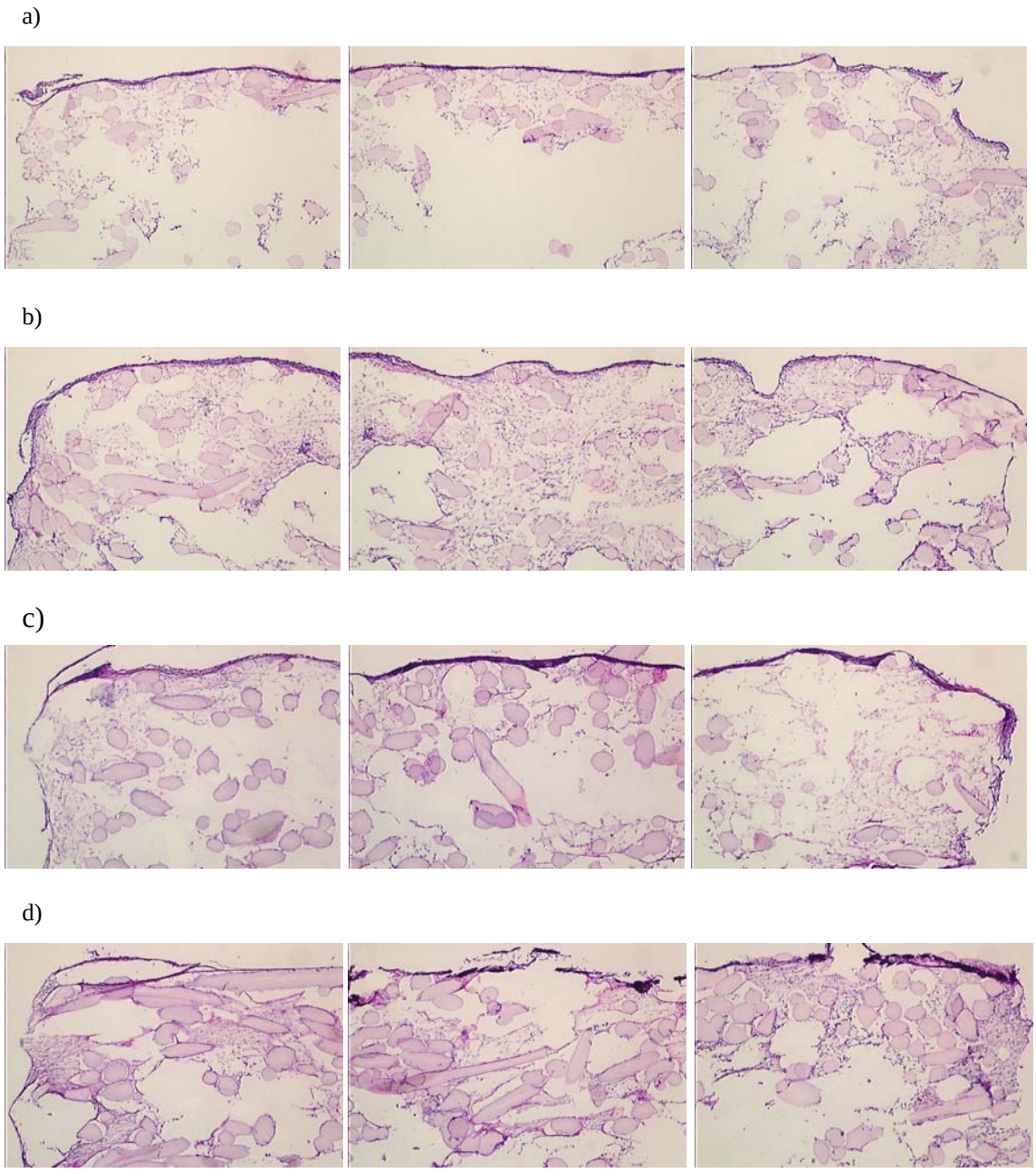

Figure VII.2. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and stained with hematoxilin and eosin: a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min.

Figure VII.3 shows images obtained from the control sections, which were incubated with PBS instead of the antibody. No positive staining was detected in these sections.

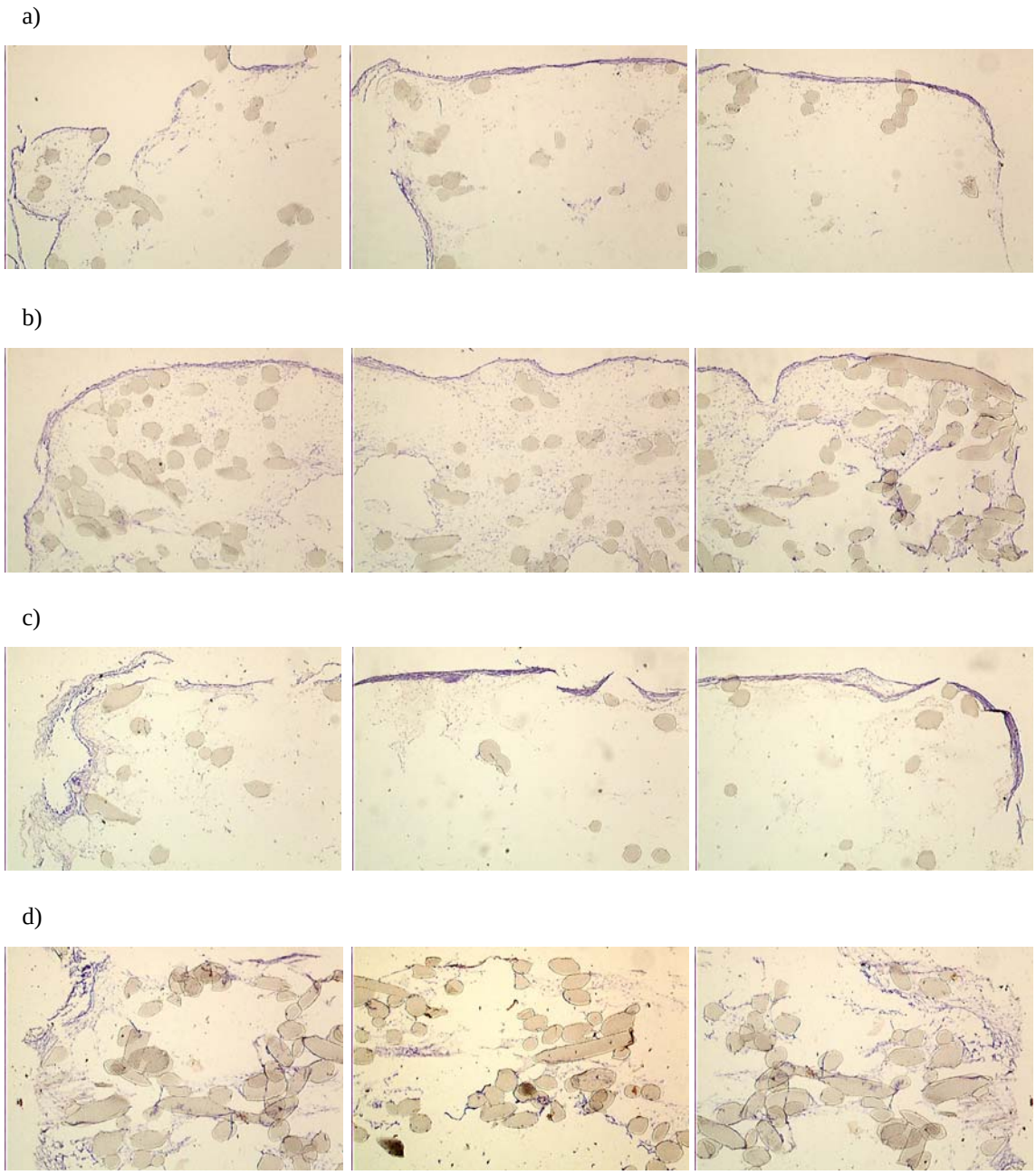

Figure VII.3. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and incubated with PBS instead of a primary antibody (controls): a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min

3.1. Vascular endothelial growth factor (VEGF)

VEGF is a polypeptide growth factor that specifically promotes the proliferation of vascular endothelial cells [10]. Studies have shown that both rodent and human osteoblast-like cells

express the mRNA for VEGF and also produce the VEGF protein [20-22]. Because blood vessels and endothelial cells are found in close proximity to osteoblasts, the VEGF produced by these cells may promote both endothelial cell proliferation and the paracrine production of other growth factors [10].

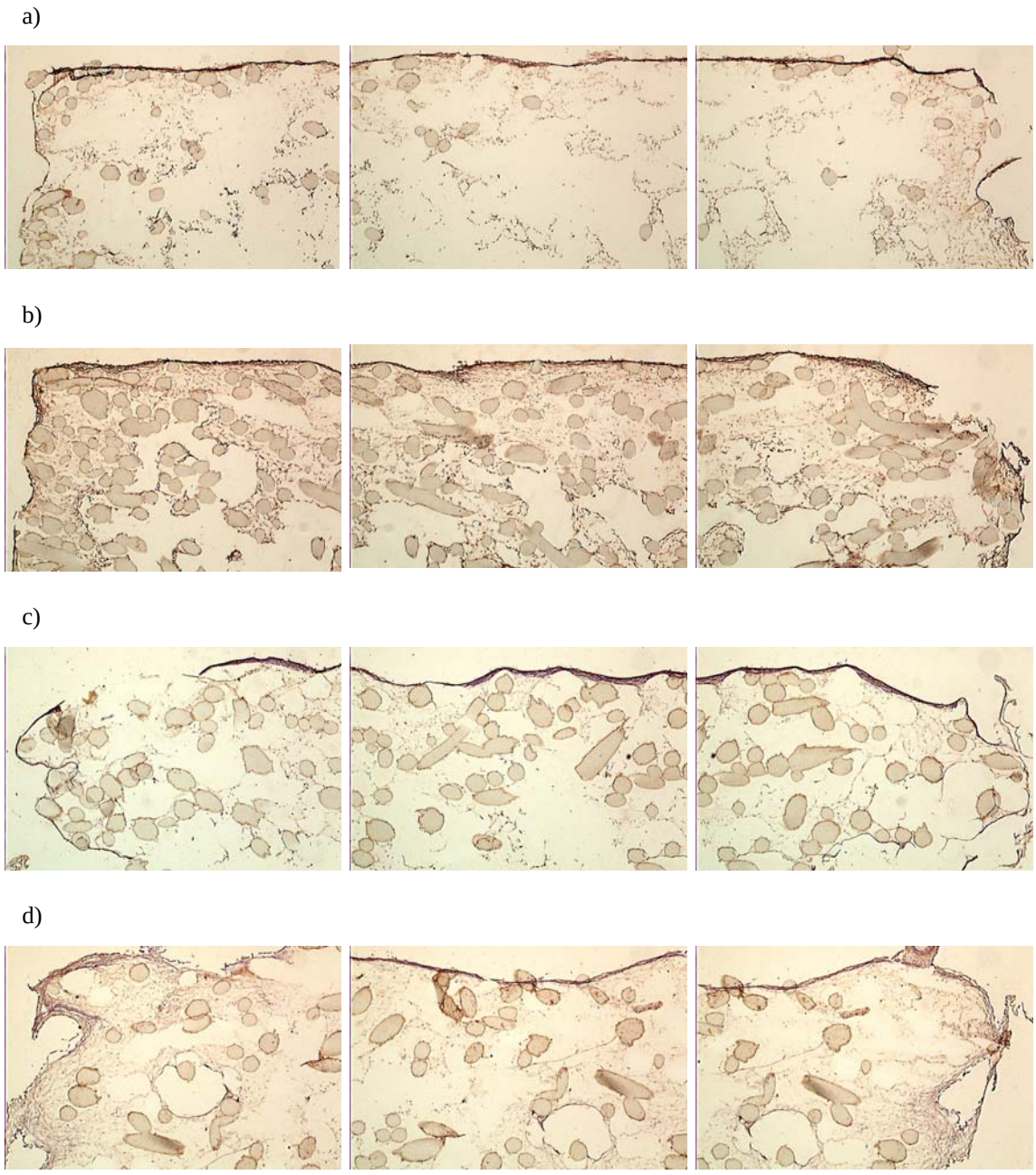

Figure VII.4. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and immunostained for VEGF: a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min

The images in figure VII.4 represent typical light microscopy pictures resulting from immunohistochemical staining for VEGF of sections obtained from samples cultured in the flow perfusion bioreactor for 10 and 16 days under two different flow rates. These images show the presence of stained areas in sections analysed for VEGF Furthermore, these images suggest an increase in the immunohistochemically stained area with increasing flow rate. This effect might be related to enhanced differentiation due to increased mechanical stimulation of the cells. It has been previously demonstrated that the increase of fluid flow in this flow perfusion bioreactor leads to enhanced differentiation and mineralization of marrow stromal cells cultured in 3-D starch-based scaffolds[13]. In addition there was also a trend of enhanced growth factor expression with culturing time, which is probably associated with increased cell and matrix content within the constructs.

The presence of VEGF in these constructs may enhance vascular tissue formation upon their implantation, increasing the viability of the transplanted cells and tissue within the scaffold. In fact, the induction of rapid vascular ingrowth has been a major limitation in bone tissue engineering [23,24], particularly when the aim is to restore a large tissue defect. This is because when a tissue engineered construct is implanted, the transplanted cells as well as the host cells that migrate into the scaffold from the native tissue depend on the transport of nutrients and waste products between the cells and the host tissue for survival; as the transport, in this first stage, is carried out exclusively by diffusion, cells that are more than several hundred microns from blood vessels in the surrounding tissue frequently either fail to engraft or die rapidly due to oxygen deprivation [25]. Therefore, the presence of VEGF in the constructs might be extremely beneficial for the development of new bone tissue as it is likely to increase the neovascularization of the scaffold/cell composite upon implantation and thus provide adequate delivery of nutrients and oxygen via the blood into the developing tissue.

3.2. Platelet-derived growth factor (PDGF-A)

Platelet derived growth factors (PDGF) have been isolated from a variety of normal and neoplastic tissues, including bone matrix and osteosarcoma cells although its original source was platelets [6]. PDGF stimulates bone DNA and protein synthesis, and may be a systemic or local regulator of skeletal growth [5,6]. As a systemic growth factor, it could be released during platelet aggregation and have important effects in the early stages of fracture healing; as a local factor, it may interact with other hormones and growth factors. In addition to its effect on bone formation, PDGF has been shown to stimulate bone

resorption, so that it appears to have a complex effect on bone remodelling [6]. Contrary to what was observed in the sections immunostained for VEGF, the sections stained for PDGF-A look similar to the control samples, which were incubated with PBS instead of the antibody, as shown on figure 5. There was no positive staining for this growth factor, demonstrating that PDGF is not expressed by marrow stromal cells cultured under the described conditions. However, it is not possible to conclude, using the presently available data, if this growth factor is expressed in this system at earlier or later stages of culture.

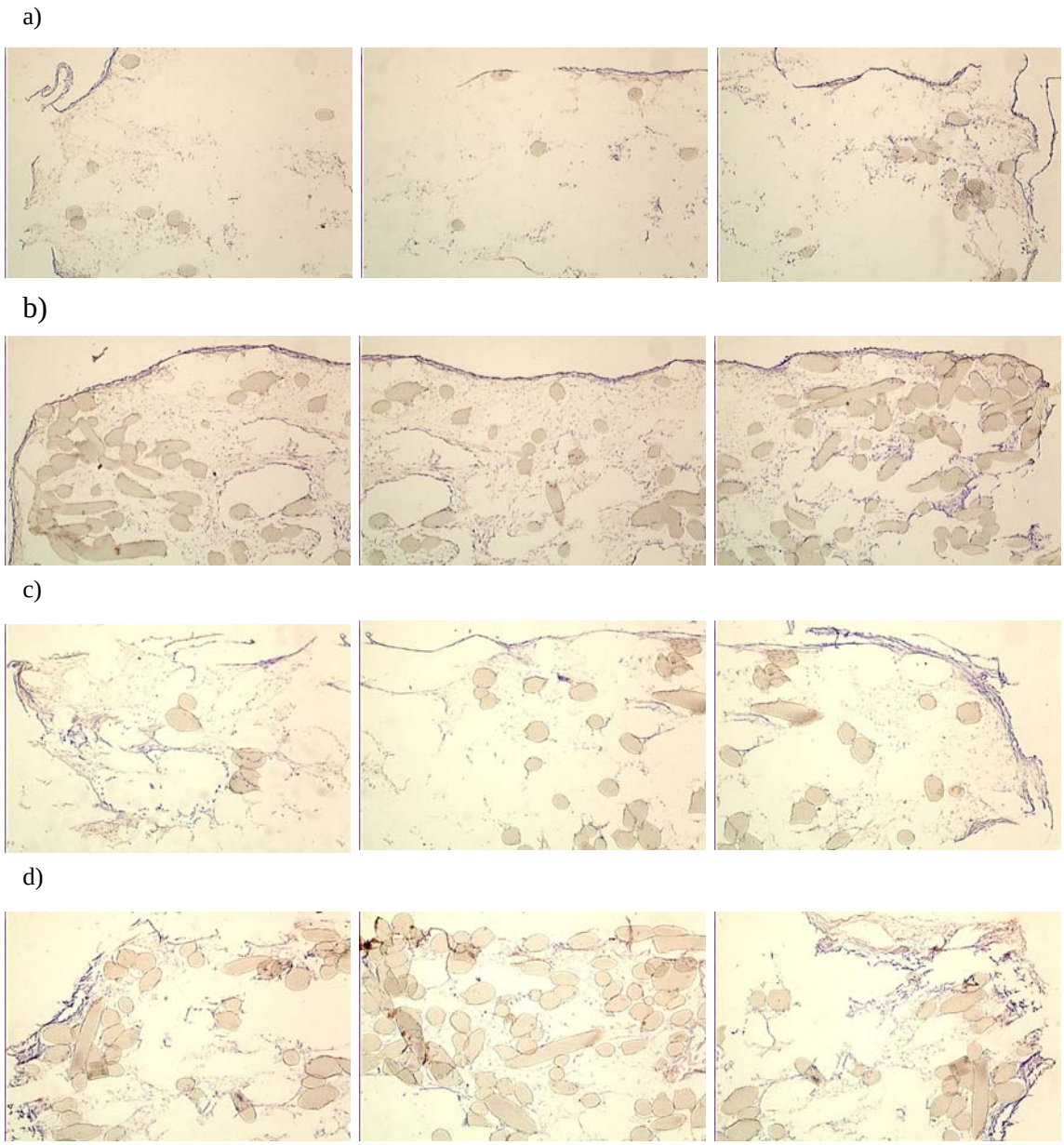

Figure VII.5. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and immunostained for PDGF-A: a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min

3.3. Transforming growth factor β1 (TGF-β1)

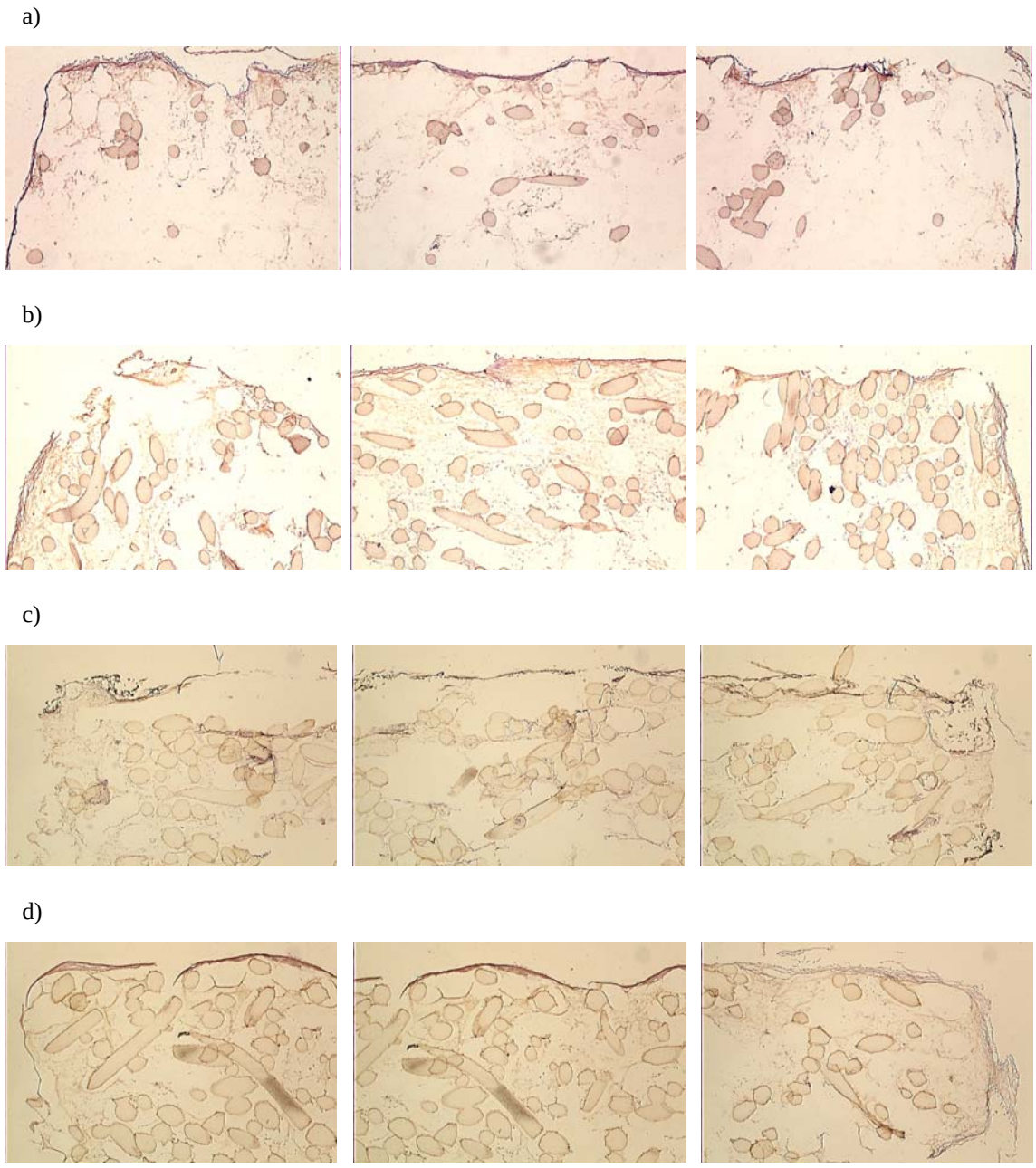

Figure VII.6. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and immunostained for TGF-β1: a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min

The transforming growth factor-β1 belongs to a family of related proteins called the TGF–β superfamily, that includes five isoforms of TGF–β and the BMPs, among others. TGF–β1 is found in many tissues but is particularly enriched in bone, platelets and cartilage[4,5]. It influences a broad range of cellular activities including growth, proliferation, differentiation and extracellular matrix synthesis and is probably the most potent multifunctional regulator of bone cell metabolism[5,6]. Previous studies suggest that occurrence of a sequential cascade of expression of members of the TGF–β superfamily during bone cell proliferation and differentiation that may influence all of the events involved in bone formation[26]. Therefore, TGF-β1, by itself or in conjunction with other growth regulators, has a major function in bone formation[6,26,27].

Figure 6 depicts images that represent typical light microscopy pictures of sections obtained from samples cultured in the flow perfusion bioreactor for 10 and 16 days under two different flow rates and immunohistochemically stained for TGF-β1. In the sections correspondent to samples cultured for 10 days at the lower flow rate, there are only some regions that are positively stained while for the remaining groups, there is widespread expression of this growth factor. This indicates that expression of this growth factor can be related to a specific period of cell development, which is enhanced by increasing the stimulation due to higher flow rate used in the perfusion bioreactor.

3.4. Fibroblast growth factor (FGF-2)

Fibroblast growth factors (FGFs) are a family of nine structurally related polypeptides which are known to play a critical role in angiogenesis and mesenchymal cell mitogenesis [4]. The most abundant FGFs in normal adult tissue are acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2). Both promote growth and differentiation of a variety of cells, including epithelial cells, myocytes, osteoblasts and chondrocytes. The mitogenic effects of FGF-1 have been associated with chondrocyte proliferation, while FGF-2 is expressed by osteoblasts and is generally more potent than FGF-1 [4].

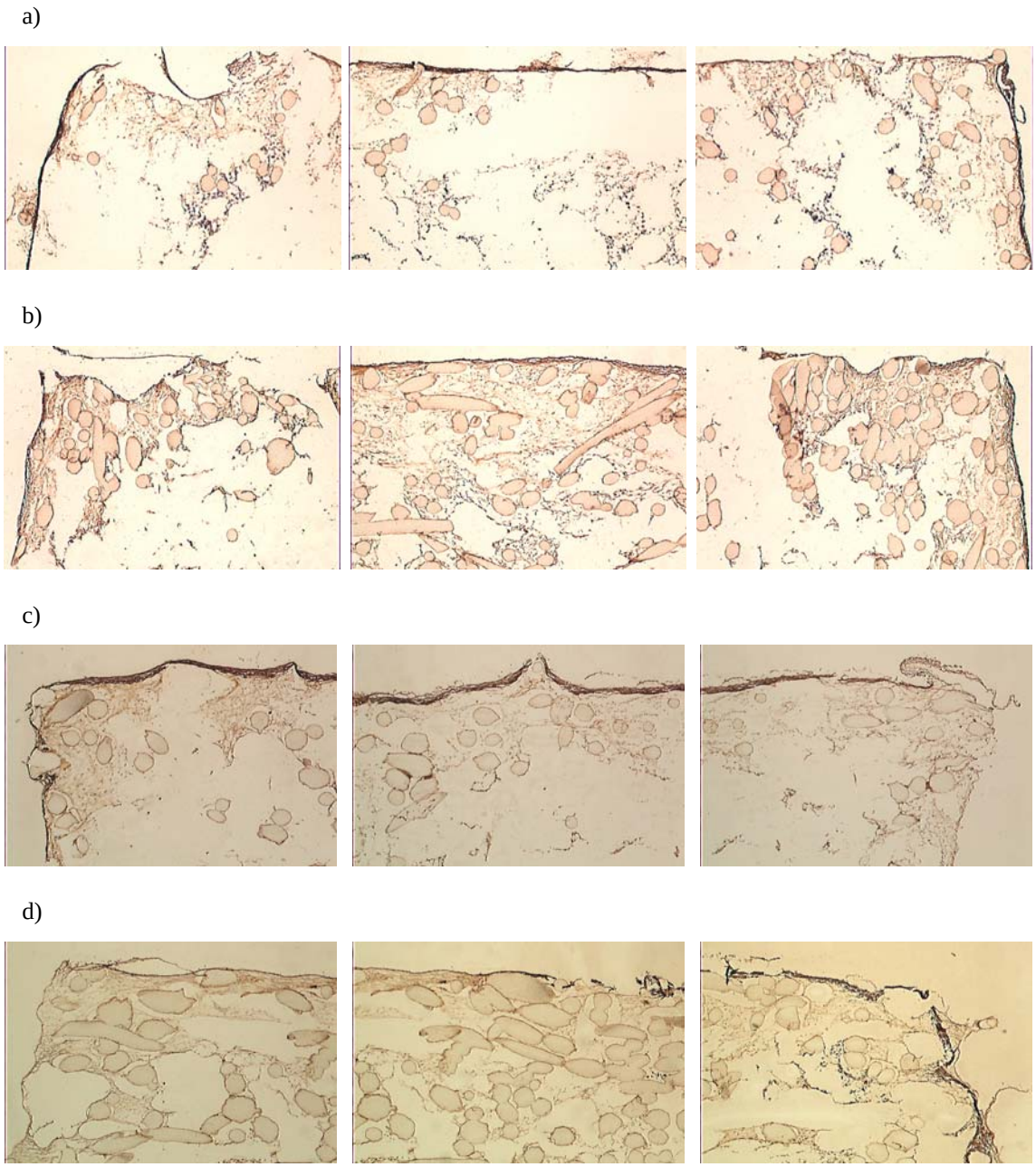

Figure VII.7. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and immunostained for FGF-2: a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min.

FGFs have mainly a proliferative effect on osteoblasts and a lesser less effect on protein synthesis. Consequently, they probably enhance bone formation by increasing the number of cells capable of synthesizing bone matrix[5,28,29]. FGF-2 can also stimulate the TGF-β synthesis by osteoblasts and may therefore exert some stimulatory effects through other

growth factors[5,28]. Like VEGF, FGFs are also angiogenic factors which are important for neovascularization during bone healing[5]. Therefore, the expression of these growth factors in the scaffold/cell constructs cultured under flow perfusion can have a positive impact on the functionality of these tissue engineered substitutes upon implantation.

The images depicting sections obtained from scaffold/cell constructs cultured under previously described conditions and stained for FGF-2, are shown in figure 7. They demonstrate the presence of several regions, corresponding to cells and matrix formed within the construct that are positively stained for this growth factor. Once again, it is visible a larger stained area in the samples cultured under higher perfusion rates, demonstrating the capability of influencing cell behaviour through the selection of different levels of mechanical stimulation provided by the flow perfusion bioreactor.

3.5. Bone morphogenetic protein (BMP-2)

Bone morphogenetic proteins (BMPs) induce the differentiation of undifferentiated mesenchymal cells into chondrogenic and osteogenic cells, and promote their differentiation [5,7]. *In vivo*, this action may result in bone generation and thus fracture repair by the newly formed bone [30]. At the cellular level, undifferentiated mesenchymal cells proliferate from the periosteum, bone marrow and muscle surrounding the fracture and begin to migrate. As a result, new bone is produced in conjunction with their differentiation into chondrogenic and osteogenic cells. In fact, it has been demonstrated that certain BMPs (BMP-2 and/or BMP-4) are present at the initial stage of this fracture healing process and seem to play an important role in several events of the bone formation cascade that leads to bone repair [1].

a)

b)

c)

d)

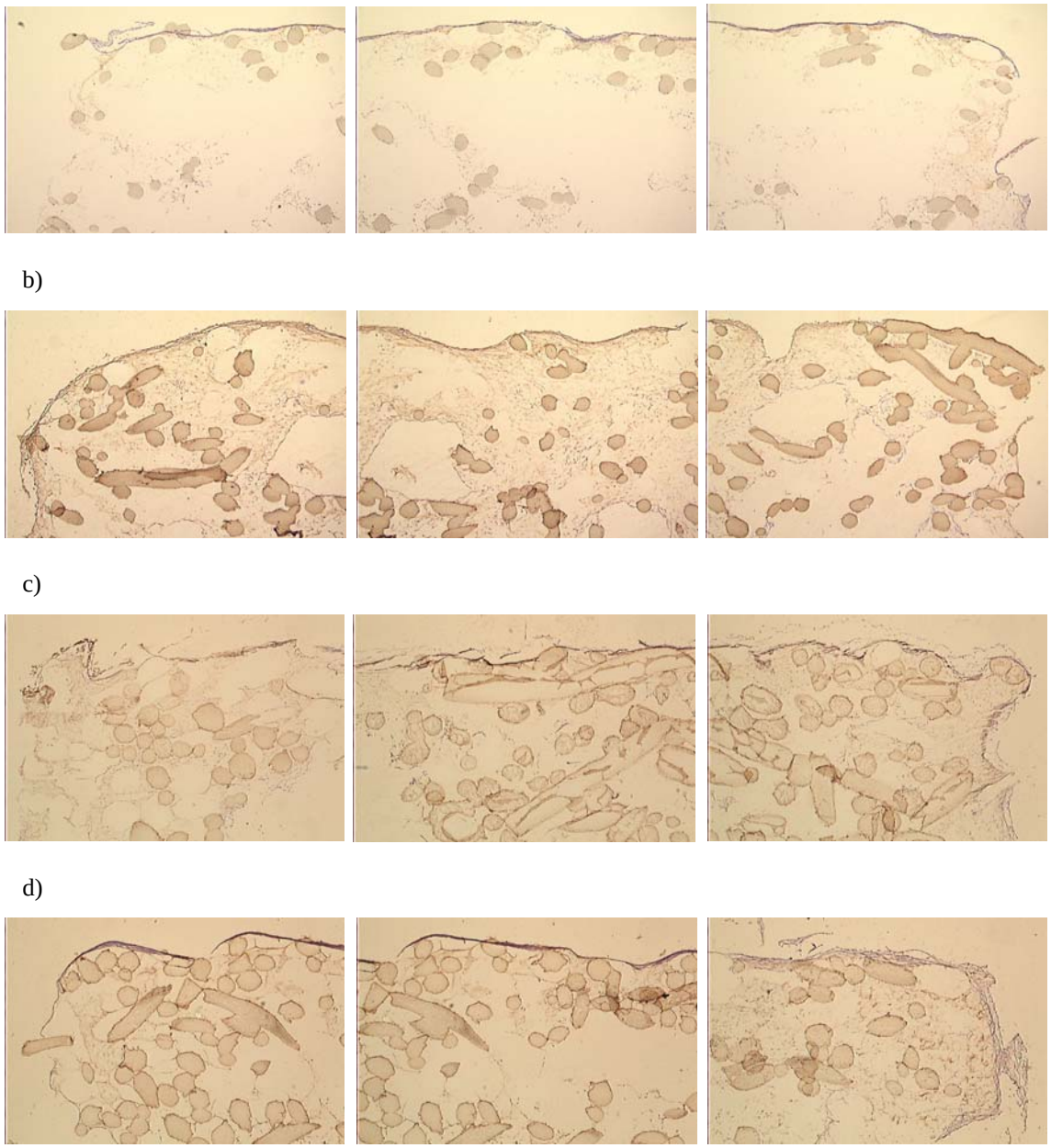

Figure VII.8. Light microscopy images (original magnification x4) of SPCL scaffold/cell constructs cultured under flow perfusion for 10 and 16 days, at two different flow rates and immunostained for BMP-2: a) Constructs cultured for 10 days at a flow rate of 0.3 ml/min; b) Constructs cultured for 10 days at a flow rate of 1 ml/min; c) Constructs cultured for 16 days at a flow rate of 0.3 ml/min; d) Constructs cultured for 16 days at a flow rate of 1 ml/min

On figure VII.8 one may see the light microscopy pictures of sections obtained from constructs cultured for 10 and 16 days in the flow perfusion bioreactor, using flow rates of 0.3 and 1 ml/min and immunohistochemical stained for BMP-2. In these pictures it is possible to identify areas positively stained for BMP-2, mostly localized in the cells and

their surrounding matrix. The sections stained for BMP-2 that correspond to samples cultured using higher flow rates, exhibit higher levels of expression of this growth factor, demonstrated by the presence on a higher number of spots/areas positively stained. This was observed in the all the samples positively analysed for the growth factors previously described, indicating that it is possible to modulate cell development by tailoring the culturing parameters. Specifically, this result suggests that higher flow rates enhance the development of rat bone marrow cells and therefore mechanical stimulation induced by this culturing parameter to the cells can be adjusted to lead to the development of tissue engineering constructs in useful (i.e., shorter) culturing times and/or to obtain bone tissue-like constructs with enhanced functionality.

Due to its capability of eliciting new bone formation, BMPs have been widely used in bone tissue engineering strategies[30,31] using a carrier device to deliver this growth factor at the implantation site. Although the efficacy of this approach in enhancing new bone formation has been shown both orthotopically and heterotopically in several different experimental animal models[30-35], the inability to find an ideal delivery system has limited the use of this approach[30,32,35].

This study shows that osteoprogenitor cells seeded in a starch-based biodegradable matrix and stimulated by flow perfusion culturing may be able to deliver growth factors, including BMP-2 after implantation, providing an osteoinductive scaffold for bone regeneration in critical size defects.

4. CONCLUSIONS

In summary, the light microscopy images obtained from 3 different regions of each of the 6 samples studied per group (culturing period/flow rate) that were immunohistochemically analysed consistently showed the presence of regions positively stained for all the growth factors examined, (namely for BMP-2, FGF-2, VEGF and TGF-β1) except for PDGF-A. These images suggest an increase in the immunohistochemically stained area with increasing flow rate, which was observed for all positively stained sections, as well as a trend of enhanced growth factor expression over culturing time. These results provide evidence that growth factors can be delivered into a scaffold via co-transplantation of cells that can naturally release them when cultured in stimulating conditions and thus accelerate

the healing and/or neotissue development upon implantation of the construct. In this sense, flow perfusion augments the functionality of scaffold/cell constructs grown *in vitro* as it combines both biological and mechanical factors to enhance cell differentiation and cell organization within the construct.

This study also shows that flow perfusion bioreactor culture of marrow stromal cells combined with biodegradable starch-based fiber meshes may constitute a useful model for *in vitro* research on the biological mechanisms associated with bone formation and regeneration. In fact, the true biological environment of a bone cell is derived from a dynamic interaction between responsively active cells experiencing mechanical forces and a continuously changing 3D matrix architecture, which can be simulated, obviously to a limited extent, in this type of bioreactor. Therefore, one can not exclude the additional influence of the biodegradable scaffold used in this system. In this case, it seems that starch-based fiber meshes support the expression of the different growth factors by rat bone marrow stromal cells cultured within these scaffolds, providing further evidence of their suitability for bone tissue engineering applications. In fact, the knowledge of how a specific scaffold material affects the gene expression of extracellular matrix molecules may lead to increased ability to customize a scaffold for a specific tissue-engineering application.

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Chapter VIII

GENERAL CONCLUSION

Chapter VIII

GENERAL CONCLUSIONS

The work developed allowed to obtain several conclusions related to the main aims of this thesis, which are summarized bellow:

i) Development of starch based scaffolds:

- It was possible to develop a wide range of processing methods to obtain starch based tissue engineering scaffolds. These methodologies allow tailoring, to an extended degree, the pore sizes and poring structure of the scaffolds as well as their degradation rates. In fact, it was shown that the degradation rates can be significantly different, depending on the processing method and on the final porosity obtained. This may allow to tailor the properties and/or porous structure of the scaffolds, according to the specific target applications within the bone tissue engineering field.
- Although only few data on mechanical properties of scaffolds for tissue engineering is found in the literature, it is possible to conclude that mechanical properties of all the tested scaffolds are very promising, when compared to scaffolds obtained from other biodegradable polymers. In addition, these properties are not significantly affected in the first 30 days of *in vitro* degradation, which suggests that the scaffold will be able to provide the necessary structural support in the first period of *in vitro* culturing and subsequent implantation.

In conclusion, it has resulted clear that the processing technologies and starch based polymers that were described present an outstanding potential for providing the adequate scaffold structures to be used in a new generation of tissue engineering strategies and may therefore constitute an important alternative to the materials currently used, providing the necessary support (from the materials science point of view) to new and under development bone tissue engineering strategies.

ii) Ability of starch based scaffolds to promote adhesion and proliferation of rat bone marrow stromal cells:

- The developed starch based scaffolds selected for the cell culturing experiments, namely the scaffolds based on SEVA-C obtained by extrusion and SPCL obtained by a fiber bonding methodology, promoted the attachment, proliferation and differentiation of rat bone marrow stromal cells. However, the SPCL fiber meshes showed increased cell proliferation due to the better interconnectivity of their porous structure.

iii) Effect of culture conditions, namely static versus flow perfusion, and scaffold's porosity on the proliferation, distribution and differentiation of RBM cells seeded onto starch based scaffolds:

- It was clearly demonstrated that the culturing conditions induce significant differences in the resulting cell-scaffold constructs. Specifically, it was demonstrated the ability of the flow perfusion bioreactor to enhance the osteogenic differentiation and the homogeneous distribution of marrow stromal cells within starch-based polymeric scaffolds, as compared to static culturing. Flow perfusion ensures fresh medium to cells, voids the accumulation of harmful metabolic products and provides cells with mechanical stimulation, providing a constant microenvironment for a high degree of cellular differentiation and better tissue development.

- It was also demonstrated (for SPCL fiber-mesh scaffolds) that increased scaffold porosity significantly enhances the proliferation of marrow stromal cells both cultured under static and flow perfusion conditions and influences the sequential development of the seeded cells. Furthermore, the flow perfusion induces *de novo* tissue modeling with the formation of pore-like structures in the scaffolds with higher porosity (75%), demonstrating that this structural aspect of scaffolding materials, in combination with the culture environment determines, to a great extent, the structure and possibly the functionality of bone-like tissue substitutes formed *in vitro*. In fact, it was shown that biodegradable starch-based fiber mesh scaffolds in conjunction with fluid flow bioreactor culture enable the creation of culture environments with minimal diffusional constraints and the ability to provide mechanical stimulation to seeded marrow stromal cells, leading to enhancement of their differentiation towards the development of a bone-like extracellular matrix and

its mineralization, forming a carbonated apatite mineral similar to the major mineral component of bone.

iv) Ability of RBM cell to express bone proteins when seeded in starch based scaffolds and cultured in a flow perfusion bioreactor:

- Immunohistochemical analysis of cell-scaffold constructs cultured in the flow perfusion bioreactor for different time periods consistently showed the presence of regions positively stained for all the growth factors examined, (namely for BMP-2, FGF-2, VEGF and TGF-β1) except for PDGF-A. The growth factor expression is enhanced with increasing flow rate due to the enhanced differentiation induced by mechanical stimulation of the cells. A trend for increased immunohistochemically stained area over culturing time was also observed. These results provide evidence that growth factors can be delivered into a scaffold via co-transplantation of cells that can naturally release them when cultured in stimulating conditions and thus accelerate the healing and/or neotissue development upon implantation of the construct. In this sense, flow perfusion augments the functionality of scaffold/cell constructs grown *in vitro* as it combines both biological and mechanical factors to enhance cell differentiation and cell organization within the construct. This study also shows that flow perfusion bioreactor culture of marrow stromal cells combined with biodegradable starch-based fiber meshes may constitute a useful model for *in vitro* studies on the biological mechanisms associated with bone formation and regeneration. In fact, the true biological environment of a bone cell is derived from a dynamic interaction between responsively active cells experiencing mechanical forces and a continuously changing 3D matrix architecture, which can be simulated, obviously to a limited extent, in this type of bioreactor. Therefore, one can not exclude the additional influence of the biodegradable scaffold used in this system. In this case, it seems that SPCL fiber meshes support the expression of the different growth factors by rat bone marrow stromal cells cultured within these scaffolds, providing further evidence of their suitability for bone tissue engineering applications.

Final concluding remarks:

In summary, microscopic, spectroscopic, biochemical and gene expression studies demonstrated that the proposed culturing system achieved enhanced proliferation and differentiation of bone marrow stromal cells, suggesting that this method achieved tissue

culture rather than cell culture. Combining this culture method with a highly porous starch based fiber mesh scaffolds scaffold has allowed for the *in vitro* development of tissue engineered constructs with high functionality, in useful periods of time.

Accordingly to all the above mentioned findings it is possible to conclude that the tissue engineering strategy proposed in this thesis, consisting in the culturing of starch based scaffolds seeded with bone marrow stromal cells and cultured in a flow bioreactor, allows to obtain a bone tissue-like substitute with high osteogenic potential for the repair of bone tissue.